Mineral and trace element nutrition in salmonids

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Aquaculture

University of Tasmania at Launceston

August, 2003

Declaration

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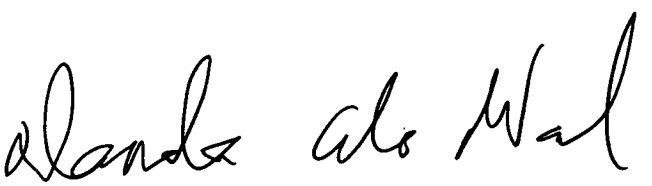
Signed 

Donald Alan Ward

"But that is the way of the scientist. He will spend thirty years in building up a mountain range of facts with the intent to prove a certain theory; then he is so happy in his achievement that as a rule he overlooks the main chief fact of all -- that his accumulation proves an entirely different thing. When you point out this miscarriage to him he does not answer your letters; when you call to convince him, the servant prevaricates and you do not get in. Scientists have odious manners, except when you prop up their theory; then you can borrow money of them."

-Mark Twain

Abstract

Minerals and trace elements are essential constituents in any diet. Our understanding of the requirements of minerals and trace elements in salmonids is limited, and based on information gained through the use of purified feeds. There exists a need to define standardised methods for determining the apparent digestibility coefficients (ADC) of minerals (calcium, iron, phosphorus, sodium, potassium, sulphur and magnesium) and trace elements (aluminium, boron, cadmium, cobalt, copper, manganese, molybdenum, nickel, selenium, vanadium and zinc), the relation between ADC and mineral retention and the effects alternative dietary protein sources, particularly those derived from plants, have on mineral and trace element ADC in salmonids. The primary objective of this research is to improve our understanding of how to best observe the nutritive value of essential minerals and trace elements and their interactions in salmonid diets. Part 1 dealt with the methodology of determining the ADC of minerals and trace elements by comparing external digestibility markers (chromium oxide and yttrium oxide) and an internal marker (acid insoluble ash) and measured the effect of faecal collection timing on ADC. High recovery marker rates and low variation in digestibility values indicated that including yttrium oxide at 0.1% effectively determined ADC for all minerals and most trace elements. Significant differences in mineral and trace element ADC were observed under the various sampling protocols, and an 18 hour collection period provided the most reliable ADC. Part 2 sought to determine the strength of relationships between digestibility and retention of minerals and trace elements and establish data for expected tissue concentrations and mineral status of salmon. There was a complex range of diet and time effects that varied between tissues. The statistical power of determining the

effect of mineral supplementation varied by element, and reflected ADC. There were no differences in growth performance between feed composed of various levels of mineral supplementation so the results provide baseline data on mineral status in relation to requirements and uptake. Part 3 dealt with the effect of plant ingredients on ADC, mineral retention, intake and growth, and compared methods of measuring maximum voluntary intake. Mineral and trace element ADC for plant ingredients differed between and amongst species. Lupin–based feeds provided growth similar to that provided by a fish meal based feed, but displayed significantly different ADC and maximum voluntary intake. The concentration of particular minerals and trace elements in plant ingredients was reflected in ADC, carcass concentrations, but not in calculated retention values.

Acknowledgements

The author gratefully acknowledges the support of the following people and agencies: Mrs Rakchanok Ward, my wonderfully supportive wife, Dr. Chris Carter, my supervisor; Dr. A Townsend for expert technical advice, assistance and the ICP-OES analysis of all samples (Central Science Laboratory, University of Tasmania, Hobart, Tasmania, Australia): the Fisheries Research and Development Corporation for financing part of this research; D. Planko, G. Kent, M. Foale, P. Cassidy, the staff of the Aquatic Centre (School of Aquaculture, University of Tasmania, Launceston, Tasmania, Australia); Dr. N . Moltschaniwskyj for her assistance in statistical analysis; J. Daniel and A. Tyson, for their administrative help, (School of Aquaculture, University of Tasmania, Launceston, Tasmania, Australia); Dr. R. Hauler, Dr. M. Bransden, Dr. M. Farhangi, Dr. K. Engin, M. Sajjadi, L. Ward, M. Flood, Dr. X. Han, T. Fox, K. Lynch, and B. Verbeeten, current and former postgraduate students within the School of Aquaculture's Nutrition group (School of Aquaculture, University of Tasmania, Launceston, Tasmania, Australia), for their assistance in these experiments; S. Pitney (Springfield Hatchery, Springfield, Tasmania, Australia) and Dr. H. King (SALTAS Hatchery, Wayatinah, Tasmania, Australia) for supplying the fish used in the experiments in this thesis; Skretting Australia (Cambridge, Tasmania, Australia), Gibson's (Cambridge, Tasmania, Australia), and M.C. Croker (Cootamundra, New South Wales, Australia) for the provision of the experimental ingredients and feeds.

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List of abbreviations

ADC	Apparent digestibility coefficient
ADCN	Apparent digestibility coefficient of the nutrient
Al	Aluminium
ANOVA	Analysis of variance
As	Arsenic
B	Boron
Ba	Barium
BW	Bodyweight
° C	Degrees Celsius
Ca	Calcium
Cd	Cadmium
Co	Cobalt
Cu	Copper
CP	Crude protein
df	Degrees of freedom
DM	Dry matter
DO	Dissolved oxygen
Fe	Iron
FER	Feed efficiency ratio
g	Gram(s)
h	Hour(s)
ICP-OES	Inductively coupled plasma optical emission spectrometry
K	Potassium

kg	Kilogram(s)
l	Litre(s)
M	Molar
Mg	Magnesium
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
Mn	Manganese
Mo	Molybdenum
Na	Sodium
Ni	Nickel
NRC	National Research Council
P	Phosphorus
P	Probability
ppm	Parts per million
®	Registered trademark name
S	Sulphur
SD	Standard deviation
Se	Selenium
SEM	Standard error of the mean
SGR	Specific growth rate
Si	Silicon
Sn	Tin
ww	Wet weight
Zn	Zinc

Chapter 1

General introduction

1.1 Feed in the salmonid industry

Salmonid production is based on the use of artificial feeds that are formulated to meet the entire dietary requirements of the fish, and can account for more than 60% of the operating costs of a salmonid production facility (Heen, 1993). The use of artificial feeds in aquaculture production is a relatively modern innovation starting with the development of moist feed pellets for salmonid production in the 1960s by researchers at Oregon State University in the United States (Willoughby, 1999). In addition to fish meal, fish oil, binders, pigments and vitamins; minerals and trace element supplements are included to provide nutritionally complete feeds (NRC, 1993; Lall, 2002). Worldwide salmonid production has increased by 1300% over the past three decades, from 3.5 MT live weight in 1970 to 48.4 MT live weight in 2000 (Figure 1.1), and is forecast to continue increasing (FAO, 2002). This growth has greatly increased the demand for salmonid feeds and given rise to concerns about the sustainability of using fish meal as the major ingredient (Hardy, 1996; Naylor et al., 2000; Tidwell & Allan, 2001). Feed and the indigestible portion of feed in fish faeces are the major components of aquaculture effluent (Cho & Bureau, 2001), and there is a need to reduce the environmental impact of aquaculture production (Hardy, 1999; Tisdell, 1999), particularly with regards to minerals, principally phosphorus (Wiesmann et al., 1988). Minerals and trace elements are important components of salmonid feeds. More needs to be known about mineral and trace element nutrition in salmonids, to provide more efficient use of aquafeed resources and promote sustainable production.

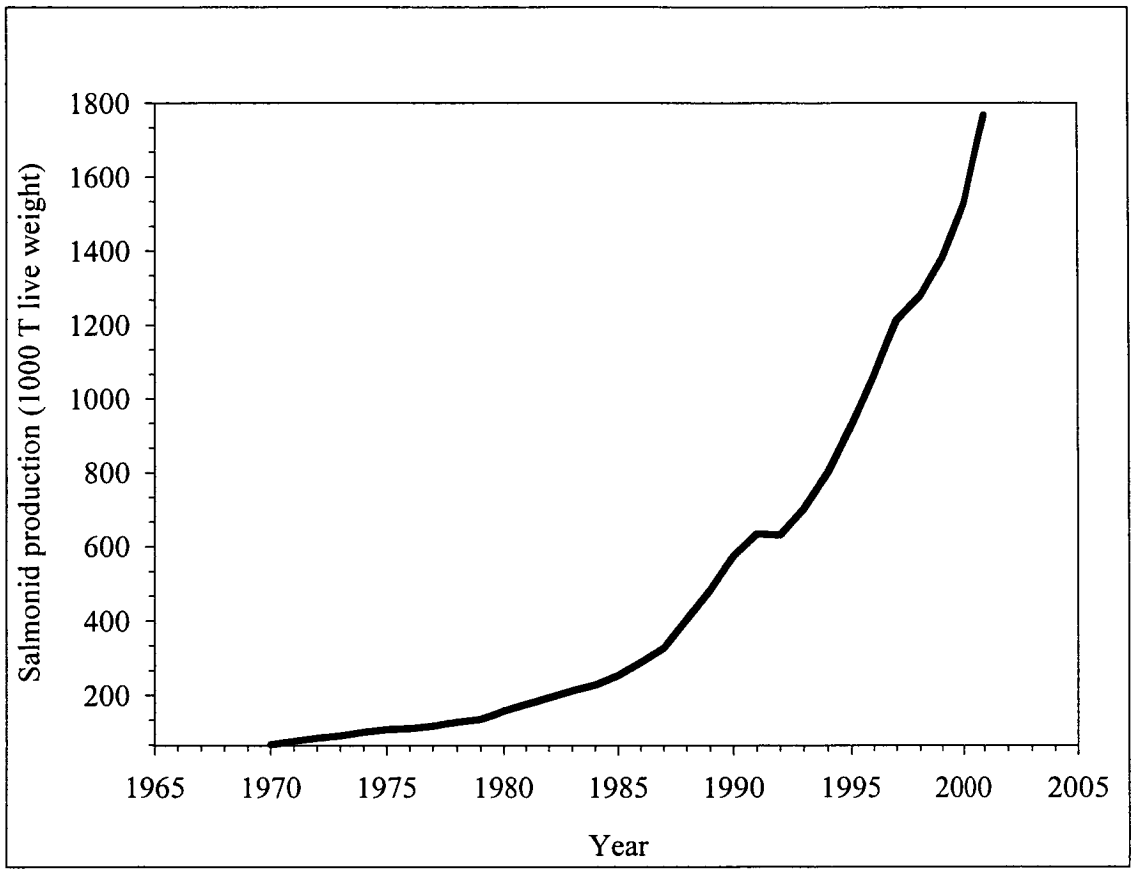

Figure 1.1 Total yearly aquaculture production of salmonids (1000 T live weight) worldwide, over the past three decades (FAO, 2002).

1.2 Mineral and trace element nutrition in salmonids

Mineral and trace element nutrition is emerging as an area of importance for aquaculture (Lall & Olivier, 1993; Watanabe et al., 1997; Lall, 2002). Minerals are vital to the health and growth of fish, but the requirements are not known with the precision of other forms of livestock. There are a number of methods of assessing the mineral nutrition of salmonids (Cho et al., 1982; Hillestad et al., 1999; Austreng et al., 2000), and salmonid feed formulations are undergoing changes driven by a number of factors (Cho & Bureau, 2001). However, little information is available regarding the effects of ingredients and supplements on mineral and trace element nutrition (Watanabe et al., 1988; Sugiura et al., 1998a).

1.2.1 Mineral and trace element requirements

Minerals and trace elements are essential components of aquafeed ingredients and are vital to the health and growth of salmonids (Lall, 2002). Minerals are important elements of a myriad of structural components, physiological processes and biochemical reactions (NRC, 1993). Trace elements are required in many enzymes, assist in the functioning of enzymes as cofactors (Watanabe et al., 1997), are integral to the immune response (Lall & Olivier, 1993) and are necessary for energy metabolism in cells of fish (Lorentzen & Maage, 1999). Recent research has provided estimates of some mineral (Table 1.1) and trace element (Table 1.2) requirements for salmonids. Salmonids can obtain minerals and trace element from two sources, feed and the aquatic environment. Salmonids can ingest sufficient amounts of calcium,

Table 1.1 Estimated mineral requirements (mg kg^{-1}) for salmonids

Element	Estimated requirement[1]	Species[2]	Source	Notes:
Calcium	3,400	RT	(Ogino & Takeda, 1978)	Reduced the digestibility of other elements
	10,000	RT	(NCR, 1993)	
	5,500	RT	(Shearer, 1995)	
Iron	60	RT	(NCR, 1993)	
	60 - 100	AS	(Waagbø et al., 1996)	
Magnesium	500	RT	(Knox et al., 1981)	
	600 - 700	RT	(Ogino et al., 1978)	
	1,400	RT	(Shearer, 1988a)	
	400	RT	(Shearer, 1995)	
	50,000	RT	(NCR, 1993)	
Phosphorus	7,800 - 8,000	RT	(Ogino & Takeda, 1978)	
	10,000	AS	(Åsgård & Shearer, 1997)	
	5,000	RT	(Shearer, 1995)	
	6,000	RT	(NCR, 1993)	
	3,700-5,300	RT	(Rodehutscord & Pfeffer, 1995)	
Potassium	7,000	RT	(NCR, 1993)	
	8,000	CS	(Shearer, 1988b)	
Sodium	6,000	RT	(NCR, 1993)	
Sulphur	Unknown			Present in feed between 1,000 – 10,000 mg kg^{-1}

[1] Estimated requirements are listed as mg kg^{-1} feed.

[2] Species of salmonid used to determine requirement: RT = rainbow trout, AS = Atlantic salmon, CS = Chinook salmon.

Table 1.2 Estimated trace element requirements (mg kg^{-1}) for salmonids

Element	Estimated requirement[1]	Species[2]	Source	Notes:
Aluminium	Unknown	AS	(Poston, 1991)	No benefit observed when supplemented up to 2,000 mg kg^{-1} in feed
Boron	Unknown			
Cadmium	Unknown	AS	(Berntssen & Lundebye, 2001)	Suggested maximum allowable concentration in feed 11 mg kg^{-1}
Chromium	Unknown			
Cobalt	Unknown	RT	(Marr et al., 1998)	Toxic at relatively low concentrations
Copper	3.0	RT	(Ogino & Yang, 1980)	
	3.0	RT	(NCR, 1993)	
	1.1	RT	(Shearer, 1995)	
	5 - 10	AS	(Lorentzen et al., 1998)	
Manganese	12 - 13	RT	(Ogino & Yang, 1978)	
	3	RT	(Shearer, 1995)	
	13	RT	(NCR, 1993)	
	15	AS	(Lorentzen et al., 1996)	
	7.5 - 10.5	AS	(Maage et al., 2000)	
Molybdenum	Unknown			
Nickel	Unknown			

Table 1.2 Continued

Element	Estimated requirement[1]	Species[2]	Source	Notes:
Selenium	0.3	RT	(NCR, 1993)	
	0.15	AS	(Poston & Combs, 1979)	
Silicon	Unknown			
Tin	Unknown			
Vanadium	Unknown			
Zinc	15 - 30	RT	(Ogino & Yang, 1978)	
	20.6	RT	(Shearer, 1995)	
	30	RT	(NCR, 1993)	
	67	AS	(Maage & Julshamn, 1993)	

[1] Estimated requirements are listed as mg kg^{-1} feed.

[2] Species of salmonid used to determine requirement: RT = rainbow trout, AS = Atlantic salmon, CS = Chinook salmon.

magnesium, sodium and potassium from seawater (Lall, 1989), but copper (Lorentzen et al., 1998; Berntssen et al., 1999), iron (Waagbø et al., 1996; Andersen et al., 1997; Maage & Sveier, 1998), manganese (Maage et al., 2000), phosphorus (Åsgård & Shearer, 1997; Baeverfjord et al., 1998), selenium (Poston & Combs, 1979; Bell & Cowey, 1989; Maage et al., 1989; Lorentzen et al., 1994), and zinc (Maage & Julshamn, 1993; Maage et al., 1993; Maage et al., 2001) must be provided in feed to prevent deficiencies in these essential elements. There are no known requirements in salmonid for the mineral sulphur, or the trace elements, aluminium, boron, cadmium, chromium, cobalt, molybdenum, nickel, silicon, tin, or vanadium, although some of these elements affect the nutritional status of other fish (Nath & Kumar, 1988) and are known to be required in livestock (Underwood, 1971; Puls, 1994).

The mineral and trace element requirements of salmonids are not known as precisely as those for other livestock. In intensive poultry production mineral and trace element requirements and mineral and trace element digestibility values are well established (NRC, 1994), allowing much more precision in formulating feeds with regard to mineral and trace element supplements. This precision is required in such intensive production systems as small changes have a relatively large impact on production costs. Therefore, it is essential that the mineral and trace element content and digestibility of a feed or feed ingredient be known with a high degree of accuracy to formulate nutritionally complete feeds that promote fish health, ensure efficient growth and reduce the environmental impact of aquaculture effluent waste. However, little information exists regard the content and digestibility of minerals and trace elements in common feed ingredients such as fish meal (Watanabe et al., 1988). Additionally, little is known of the correlation between digestibility and the

bioavailability or retention of minerals in the various bodily organs of fish (Satoh et al., 1987b; Nordrum et al., 1997; Baudin et al., 2000).

1.2.2 Supplementation

Commercial salmon feeds are often supplemented with mineral pre-mixes, usually in excess of requirements (Lall, 1989), but this practice of over-supplementation must cease as Atlantic salmon production reaches commodity status and the cost of feed per unit of production becomes increasingly important (Hardy, 2000). Mineral pre-mixes include calcium, phosphorus, potassium, sodium, magnesium, iron, copper, zinc, molybdenum, manganese, selenium, iodine and vitamin B_{12} supplements which contain cobalt. Minerals and trace elements will become the limiting factor in growth as limitations in macronutrient requirements are met and exceeded, particularly in salmonids which have high growth and feed conversion rates.

1.2.3 Assessing mineral and trace element nutrition in salmonid feeds

There are numerous reasons for accurately assessing mineral and trace element nutrition in salmonids. Improving our knowledge of mineral and trace element digestibility will provide economic benefits for farmers and feed suppliers (Tisdell, 1999). The digestibility of feed ingredients (Cho & Slinger, 1993) and changes in feed formulations are the main factors affecting the amount of fish meal required by production systems and the composition of aquaculture waste output (Cho et al.,

1994; Cho & Bureau, 2001). Replacing fish meal with alternative forms of protein requires changes in mineral supplementation (Ketola, 1975), and an assessment of the digestibility of the minerals provided by these replacement ingredients (Storebakken et al., 1998a; Sugiura et al., 1998a).

1.2.4 Apparent digestibility coefficients

The apparent digestibility coefficients (ADC) of macronutrients in feed ingredients have been calculated by comparing the concentration of an indigestible marker, often a metal oxide that has been added to a feed, within feed and faecal samples with the concentrations of a nutrient in those same samples (Austreng, 1978; Cho & Slinger, 1979; Austreng et al., 2000). Only recently have researchers identified a need for a standard method of assessing the digestibility of macronutrients in commercial salmon feeds (Hillestad et al., 1999). As yet there is no standard method for determining or verifying the digestibility of micronutrients in commercial feeds. A number of factors associated with significant differences in the digestibility of macronutrients, and some micronutrients, have been identified in finfish, including: the genetic makeup of the fish (Thodesen et al., 2001), conditions under which the fish are kept (Storebakken et al., 1998b; Sorensen et al., 2002), differences in feed and feed processing (Cheng & Hardy, 2003), the methods of feeding and faecal collection (Windell et al., 1978; Hajen et al., 1993a; Storebakken et al., 1998a; Percival et al., 2001), differences in processing these samples, differences in type (Austreng et al., 2000; Carter et al., 2003) and concentration of the digestibility marker, differences in processing and decomposition of the samples prior to analysis (Scott, 1978),

differences in methods of analysing the nutrient and marker content of these samples (Leid et al., 1982), and researchers have a choice of at least two different formula to use when calculating the digestibility for ingredients (Cho & Slinger, 1979; Sugiura et al., 1998b; Forster, 1999). There are a variety of faecal collection methods used in digestibility studies in salmonids including: anal suction, dissection, mechanical sieving, settlement and stripping (Table 1.3), each differing
with regard to the effect on calculating ADC, from either leaching or alterations to the normal digestive process. ADC values can also be less than 0% and greater than 100%. Negative ADC result when concentrations of a nutrient in the faeces are greater than the concentration in the feeds that produced those faeces. Faecal nutrient concentrations can be increased by the excretion of minerals from the fish obtained from drinking, absorption of minerals through the gills, and/or the mobilisation of bodily reserves, and from contamination from minerals in the water surrounding the faecal sample, depending on the type of method used to obtain the sample. ADC values greater than 100% can result when assessing an ingredient by substituting it for a proportion of a reference feed and the experimental feed possesses a digestibility greater than the reference feed. This indicates that assuming the digestibility of nutrients in the reference feed is constant and not affected by the inclusion of test ingredients is not justified, and the ADC of a nutrient within the reference portion of a feed can be recalculated to assess the antagonistic or synergistic effects on the basal portion of experimental feeds (Sugiura et al., 1998a).

Table 1.3 Methods of faecal collection in digestibility research for salmonids

Method of collection[1]	Source
Anal suction	(Windell et al., 1978; Percival et al., 2001)
Dissection	(Austreng, 1978; Riche et al., 1995; Riche & Brown, 1996; Rosjo et al., 2000)
Hand-netting	(Windell et al., 1978)
Mechanical sieving	(Choubert et al., 1982; Burel et al., 2000)
Settlement	(Cho et al., 1975; Cho & Slinger, 1979; Cho et al., 1982; Atkinson et al., 1984; Satoh et al., 1992; Gudmundsson et al., 1995; Sugiura et al., 1998a; Morales et al., 1999; Carter & Hauler, 2000; Sugiura et al., 2000c; Zhu et al., 2001; Cheng & Hardy, 2002a, b; Green et al., 2002b; Lanari & D'Agaro, 2002; Yamamoto et al., 2002; Carter et al., 2003; Chen et al., 2003; Thiessen et al., 2003)
Settlement and dissection	(Kabir et al., 1998)
Settlement and stripping	(Sugiura et al., 1998a)
Settlement, stripping and mechanical sieving	(Vandenberg & de la Noüe, 2001)
Settlement, stripping and dissection	(Hajen et al., 1993a; Hajen et al., 1993b)
Stripping	(Tacon & Rodrigues, 1984; Dong et al., 1993; Aksnes et al., 1996; Refstie et al., 1997; Aksnes & Opstvedt, 1998; Baeverfjord et al., 1998; Refstie et al., 1998; Storebakken et al., 1998a; Storebakken et al., 1998c; Vielma & Lall, 1998; Hillestad et al., 1999; Refstie et al., 1999; Rodehutscord & Pfeffer, 1999; Thodesen et al., 1999; Austreng et al., 2000; Rodehutscord et al., 2000; Storebakken et al., 2000; Sugiura et al., 2001)
Stripping and mechanical sieving	(Vens-Cappell, 1985; Weatherup & McCracken, 1998)

[1] Settlement is often referred to as the "Guelph" system or column settlement, and mechanical sieving is often referred to as the "Choubert" method or the tank method.

with regard to the effect on calculating ADC, from either leaching or alterations to the normal digestive timing.

1.2.3 Changes in feed formulation

The formulation of salmonid feeds has been changing over the past decade in response to a number of factors. Fish meal continues to be the primary source of protein used in salmonid feeds, although price variability, demands from terrestrial livestock feed producers, and other economic and environmental factors have significant effects on the price and availability of fish meal to aquafeed producers (Hardy, 1996). For these reasons recent research has focused on identifying alternative sources of protein for salmonid feeds (Bureau et al., 2000; Carter & Hauler, 2000; Sugiura et al., 2000a), and assessing the effect of these ingredients on the growth and health of salmonids (Farhangi & Carter, 2001). However, little research has been conducted regarding the effect of including these different ingredients on the mineral content and digestibility of minerals in the resulting feeds (Sugiura et al., 1998b). Phosphorus is the notable exception (Riche & Brown, 1996; 1999; Rodehutscord et al., 2000). The effect of phosphorus supplementation and the phosphorus content of feed and feed ingredients has been investigated more thoroughly than other elements (Ketola, 1985; Lall, 1991; Riche & Brown, 1996; 1999).

1.3 Aims of this study and outline of the thesis

This study investigated various aspects of mineral and trace element nutrition in Atlantic salmon (*Salmo salar*, L.). The thesis consists of three parts: Part one, (Chapters 2 and 3) investigated the use of markers and faecal collection; Part two (Chapters 4 and 5) dealt with mineral supplementation, retention and the effects of citric acid on mineral availability; Part three (Chapters 6 and 7) dealt with the effect of plant protein meal inclusion on mineral nutrition, growth and feed intake.

1.3.1 The effects of sampling and analytical methods on ADC calculations

The first part of the thesis identified a method of faecal collection that provided the required amount of sample, and investigated the effects of sampling methods on mineral and trace element ADC calculations. The first and second experiments (Chapter 2) considered the use of various concentrations of the external markers chromium oxide and yttrium oxide, the internal marker acid insoluble ash and the effect of sample collection timing on determining the ADC of mineral and trace elements. The third experiment (Chapter 3) contrasted three different faecal sample collection methods and their effects on ADC values. Research has identified the effect of faecal collection methods on macronutrient ADC (Windell et al., 1978), but no information exists describing the effects, if any, the method of faecal collection may have on the calculation of mineral and trace element ADC. A number of compounds have been identified as useful digestibility makers for calculating the ADC of macronutrients (Riche et al., 1995; Kabir et al., 1998; Austreng et al., 2000;

Carter et al., 2003), but it is unknown if the type of marker compound or level of inclusion in the feed affects mineral and trace element ADC values. Additionally, there is no information comparing the rate of passage for external digestibility makers with minerals and trace elements, which affects ADC values for macronutrients (Austreng et al., 2000).

1.3.2 The effects of supplementation on mineral nutrition

The second part of this thesis observed the effects of mineral supplementation on changes in ADC over time, mineral retention in muscle, blood, liver and kidney samples, the relationship between ADC and mineral retention in tissue samples (Chapter 4), and the use of citric acid to alter mineral digestibility (Chapter 5). ADC are often calculated from faecal samples obtained from faecal collections taken at the end of an experiment (Austreng, 1978; Storebakken et al., 1998a; Sugiura et al., 1999). The effect of mineral supplementation over time on mineral and trace element ADC is unknown, as is the relationship between ADC and the mineral content of the body tissues of Atlantic salmon. There are known to be interactions between minerals and other minerals and minerals and vitamins (Lall, 2002). However, the effect of dietary mineral content on the digestibility of other minerals is limited (Satoh et al., 1987a; Vielma & Lall, 1998), and the relationship between dietary mineral concentrations and the digestibility of trace elements needs to be clarified. The addition of dietary supplements alter the digestibility of minerals and trace elements in rainbow trout (Sugiura et al., 1998a), and may have a beneficial effect on the digestibility of minerals and trace elements in Atlantic salmon. Citric acid increased

the availability of phosphorus, calcium, strontium, zinc, copper, iron and magnesium in rainbow trout (*Oncorhynchus mykiss*) (Sugiura et al., 1998a). Organic forms of zinc, zinc-amino acid complexes, have proven to be more effective than inorganic zinc sulphates at supplying this trace element to channel catfish (*Ictalurus puntatus*, L.) (Paripatananont & Lovell, 1995), and it may be possible to use metal-amino acid supplements in salmonids. Mineral requirements in salmonids are often assessed using whole fish (Shearer et al., 1994; Åsgård & Shearer, 1997; El-Mowafi et al., 1997; Lorentzen & Maage, 1999; Storebakken et al., 2000; Sugiura et al., 2000b; Green et al., 2002a; 2002b; Apines et al., 2003). However, some tissues present in whole fish, such as scales, can greatly influence the mineral concentration of a sample, and the analysis of discrete tissues samples such as blood, muscle, and liver-kidney samples would aid in distinguishing if trace element deficiencies exist.

1.3.3 The effect of novel ingredients and feed intake on mineral nutrition

Part three analysed the effect of substituting plant ingredients for a portion of the protein provided by fish meal on mineral and trace element ADC values (Chapter 6) and the effect of high levels of lupin inclusion on mineral and trace element ADC, fish growth parameters, feed intake and mineral retention (Chapter 7). Little information exists regarding the mineral digestibility of feeds composed of fish meal or the effect of replacing the fish meal portion of a feed with plant proteins on mineral and trace element digestibility. Information regarding the requirements and availability of minerals and trace elements has been derived using feeds composed of purified ingredients, and purified feeds are not used for production purposes.

Therefore, it is necessary to understand the effects of common feed ingredients on our methods of mineral and trace element analysis. Most feed formulations assume that the solubility of a supplement is not altered by the other primary ingredients in a feed. There are a number of anti-nutritional compounds in feed ingredients, particularly plant ingredients used to replace fish meal, that affect the digestibility of phosphorus and other nutrients, including phytate, non-starch polysaccharides, oligosaccharides and other carbohydrates (Glencross et al., 2003). The effect these anti-nutritional compounds have is yet to be determined for most minerals and trace elements. There may also be differences within a species of plant that could affect the mineral digestibility of a feed even though the ingredients display similar macronutrient properties.

Each chapter of this thesis has been prepared as a distinct submission to a peer-reviewed journal. Therefore, some repetition, particularly in the materials and methods sections, will be present.

1.3.4 Summary of aims

The aims of this thesis are as follows:

- Identify a method of faecal collection that provided the required amount of sample;
- Investigate the effects of sampling methods on mineral and trace element ADC calculations;
- Observe the effects of mineral supplementation on changes in ADC over time;

- Observe the effect of mineral supplementation on mineral retention in muscle, blood, liver and kidney samples;
- Observe the relationship between ADC and mineral retention in tissue samples
- Assess the effectiveness of citric acid to alter mineral digestibility;
- Analyse the effect of substituting plant ingredients for a portion of the protein provided by fish meal on mineral and trace element ADC values;
- Assess the effect of high levels of lupin inclusion on mineral and trace element ADC, fish growth parameters, feed intake and mineral retention.

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Chapter 2

A comparison of the effectiveness of external (yttrium oxide and chromic oxide) and internal markers (acid insoluble ash) and timing of faecal collection for determining the apparent digestibility coefficients of minerals and trace elements in Atlantic salmon (*Salmo salar*, L.) feeds

Abstract

Effectiveness of two external markers, an internal marker and faecal collection sampling period for determining the apparent digestibility coefficients (ADC) of minerals and trace elements within Atlantic salmon feeds were compared. The external markers, yttrium oxide (Y_2O_3), and chromium oxide (Cr_2O_3), were compared at inclusions of 1.0, 0.1, 0.01, and 0.001% ww of the feeds and with the internal marker acid insoluble ash (AIA). After wet decomposition with concentrated nitric acid, samples were analysed for a range of mineral and trace elements via inductively coupled plasma optical emission spectroscopy. The method proved ineffective for chromium determination, and it was necessary to reanalyse with an alternative optical density method. Yttrium oxide displayed many advantages over chromium oxide and AIA for determining mineral and trace element ADC, and the inclusion level of 0.1% provided the most reliable results. External markers in general may not be accurate for measuring "ultra-trace" elements and the information gained via ICP-OES may not represent the true bioavailability of some elements. The effect of faecal sampling period on mineral and trace element ADC was compared using the feed marked with 0.1% yttrium oxide. Faeces were collected over three different time periods within a 24 h period: 4 x 6 h, 2 x 12 h, and 1 x 24 h. Magnesium, manganese, phosphorus, iron and chromium displayed significant differences in ADC relating to sampling period. An inclusion level of 0.1% yttrium oxide and faecal sampling over at least an 18 h period after feeding proved to be the most effective method of determining mineral and trace element ADC in Atlantic salmon feed.

Keywords: Aquaculture feeds; Atlantic salmon; digestibility markers; mineral and trace element digestibility

2.1 Introduction

Aquaculture production continues to increase rapidly worldwide, and these increases create a demand for efficient and environmentally sustainable aquafeeds (Cho & Slinger, 1993; Hardy & Green, 1999; Tidwell & Allan, 2001). Determining the digestibility of minerals and trace elements within salmonid feeds is crucial to providing feeds that are both efficient at promoting growth and limiting environmental impact. The mineral composition of salmonid feed ingredients is highly varied (Watanabe et al., 1988; NCR, 1993). In addition, routine changes in feed formulations and increasing need to find suitable ingredients, depending on the cost and availability of ingredients, result in variations in the mineral composition of salmonid feeds (Naylor et al., 2000). Commercial salmonid feeds are usually supplemented with mineral and trace element pre-mixes in excess of requirements to prevent nutritional deficiencies (Hilton, 1989; Watanabe et al., 1997). To improve feed efficiency and reduce environmental impacts, mineral availability should more closely match requirements and more attention be paid to possible mineral interactions in the feed (Cho & Slinger, 1993; Watanabe et al., 1997; Tisdell, 1999). Determination of mineral and trace element digestibility is the first stage in achieving these goals.

The most used method of determining the apparent digestibility coefficients (ADC) of feed or ingredients is indirectly via the inclusion of markers within feed (Austreng, 1978; Cho & Slinger, 1979; Austreng et al., 2000). To be effective, markers must be inert, not be absorbed or interact with the animal's digestive system or other components of the feed, and not alter the passage of nutrients through the gastro-

intestinal tract or the metabolism of the fish (Kotb & Luckey, 1972; Austreng, 1978; NCR, 1993; Austreng et al., 2000). In the past, chromium oxide (Cr_2O_3) was the marker of choice (Austreng, 1978; Aksnes et al., 1996), but recently various metal oxides, particularly yttrium oxide (Y_2O_3) and ytterbium oxide (Yb_2O_3), have been used (Riche et al., 1995a; Sugiura et al., 1998a; Hillestad et al., 1999; Austreng et al., 2000).

The effectiveness of chromium oxide (Austreng, 1978; Windell et al., 1978) and yttrium oxide (Sugiura et al., 1998a; Hillestad et al., 1999), for determining ingredient ADC in salmonid feeds have been evaluated, but little information exists on the effectiveness of these markers for determining the digestibility of minerals and trace elements. Furthermore, (Sugiura et al., 1998a) calculated a 4.8 ± 1.7% apparent absorption of chromium oxide based on yttrium oxide as the marker. Recently, Austreng et al. (2000) evaluated a wide range of trivalent metal oxides, and determined that lanthanum oxide (La_2O_3), yttrium oxide, and ytterbium oxide are acceptable substitutes for chromium oxide, and can be used at lesser concentrations to estimate the ADC of nitrogen and fat in salmonids. Hillestad et al. (1999) calculated a variety of ADC values in Atlantic salmon, for phosphorus using lanthanum oxide, yttrium oxide, and chromium oxide, and concluded that, due to the differences between calculated phosphorus ADC, a more accurate method was required, but did not clarify further or suggest a specific analytical method.

The effectiveness of a digestibility marker for aquafeeds should be assessed on the rate of marker recovery in the feed and faeces (Hillestad et al., 1999; Austreng et al., 2000), uniformity of passage through the digestive system (Leid et al., 1982; Austreng

et al., 2000; Vandenberg & de la Noüe, 2001), accuracy of calculated ADC (Tacon & Rodrigues, 1984; Riche et al., 1995b; Hillestad et al., 1999; Morales et al., 1999; Austreng et al., 2000), marker solubility (Austreng et al., 2000), ease of analysis (Atkinson et al., 1984), any interactions the marker may have with other ingredients in the feed or the digestive system of the fish (Shiau & Liang, 1995; Ng & Wilson, 1997; Shiau & Shy, 1998; Fernandez et al., 1999).

The aim of this experiment was to identify an effective method of determining the ADC of mineral and trace elements within Atlantic salmon feeds using standard methods for preparing samples for elemental analysis, and assess the suitability of various concentrations of the external markers chromium oxide and yttrium oxide with an internal marker acid insoluble ash for calculating mineral and trace element ADC. Those elements commonly referred to as trace elements are found in concentrations of less than 100 mg kg^{-1} (Vandecasteele & Block, 1993), and in this experiment were aluminium, arsenic, boron, barium, cadmium, cobalt, copper, manganese, molybdenum, nickel, lead, selenium, silicon, tin, vanadium, and zinc, and the remainder, calcium, iron, potassium, magnesium, sodium, phosphorus, and sulphur, are referred to as minerals.

2.2 Materials and methods

2.2.1 Experiment 1 – marker comparisons

2.2.1.1 Feed

Eight experimental feeds were formulated from a reference salmon feed, to contain 0.001%, 0.01%, 0.1% or 1.0% wet weight of either yttrium oxide or chromium oxide, but not both. The markers were mixed into a moist reference feed (Table 2.1). All dry ingredients were added to the fish meal separately. The liquid vitamin E supplement was mixed thoroughly with the fish oil and both were added to individual batches of dry mix, and thoroughly mixed. The base salmon feed was formulated to meet the mineral and trace element requirement estimates from various sources (see Chapter 1, Table 1.1 and 1.2) and contained sufficient concentrations of minerals (Table 2.2) and trace elements (Table 2.3). Immediately prior to pelleting 100 ml distilled water kg^{-1} dry weight feed was added, while mixing. The feeds were pelleted at room temperature with a 3.4 mm die, on a California Laboratory Pellet Mill (CL-2 laboratory pellet mill, California Pellet Mill Co., San Francisco, U.S.A.). Pelleting proceeded from the feed with the lowest concentration of marker to the highest. After finishing all the experimental feeds for one marker, the pellet mill was cleaned of any residual marked feed. The pelleted feeds were oven-dried at 40 °C for over 24 h, and stored in a cold room at 2.7 °C.

Table 2.1 Ingredient composition of the base salmon feed

Ingredients	Source[1]	g kg^{-1} feed
Fish meal	Skretting	700
Fish oil	Skretting	150
Wheat flour	Gibson's	60
CMC binder[2]	Sigma-Aldrich	10
Mineral supplement		mg kg^{-1} feed
Potassium phosphate dibasic	Sigma-Aldrich	60000
Calcium carbonate	Sigma-Aldrich	7000
Sodium chloride	Sigma-Aldrich	10000
Magnesium carbonate	Sigma-Aldrich	700
Ferrous sulphate	Sigma-Aldrich	200
Zinc sulphate	Sigma-Aldrich	75
Manganous sulphate	Sigma-Aldrich	80
Cupric sulphate	Sigma-Aldrich	23.6
Cobalt sulphate	Sigma-Aldrich	9.5
Potassium iodide	Sigma-Aldrich	1.4
Sodium selenate	Sigma-Aldrich	0.7
Vitamin supplement		mg kg^{-1} feed
Choline chloride	Sigma-Aldrich	1330
myo-Inositol	Sigma-Aldrich	300
DL alpha tocopherol acetate	Sigma-Aldrich	175
Stay-C® (L-ascorbyl 2 polyphosphate)	Roche	50
Calcium D-pantothenate	Sigma-Aldrich	22
Nicotinic acid	Sigma-Aldrich	10
Retinol acetate (2800000 IU/g)	Sigma-Aldrich	4.8
Riboflavin	Sigma-Aldrich	4
Pyridoxine HCl	Sigma-Aldrich	3.7
Vitamin D_3 powder (850000 IU/g)	Sigma-Aldrich	6
Menadone sodium bisulphate	Sigma-Aldrich	2
Thiamin HCl	Sigma-Aldrich	1.1
Folate	Sigma-Aldrich	1
d-Biotin	Sigma-Aldrich	0.15
Vitamin B_{12}	Sigma-Aldrich	0.01

Reference diet formulated as per Carter and Hauler (2000).

[1] Ingredients from Skretting and Gibson's were sourced from Cambridge, Tasmania, those from Sigma-Aldrich sourced from Castle Hill, NSW and Stay-C® was from Roche Vitamins Australia, Frenchs Forest, NSW.

[2] Markers were substituted for the CMC binder to produce the experimental feeds.

Table 2.2 Mean (± SEM, n = 3) mineral concentrations (mg kg^{-1}) of the experimental feeds.

Marker	Chromium				Yttrium			
Conc.	0.001%	0.01%	0.1%	1.0%	0.001%	0.01%	0.1%	1.0%
Ca	23,617	181,198	25,968	111,515	24,788	1,062,251	25,675	218,649
	(181,198)	(25,968)	(111,515)	(24,788)	(1,062,251)	(25,675)	(218,649)	(21,409)
Fe	423	209	352	90	373	235	372	110
	(209)	(352)	(90)	(373)	(235)	(372)	(110)	(411)
K	26,943	51,303	26,961	87,482	26,657	27,434	27,962	588,744
	(51,303)	(26,961)	(87,482)	(26,657)	(27,434)	(27,962)	(588,744)	(24,797)
Mg	2,244	16	2,344	145	2,318	953	2,303	258
	(16)	(2,344)	(145)	(2,318)	(953)	(2,303)	(258)	(2,150)
Na	11,452	1,099	11,346	12,307	11,413	9,473	11,802	58,885
	(1,099)	(11,346)	(12,307)	(11,413)	(9,473)	(11,802)	(58,885)	(10,557)
P	32,956	46,130	32,494	113,983	32,746	59,288	33,578	814,524
	(46,130)	(32,494)	(113,983)	(32,746)	(59,288)	(33,578)	(814,524)	(30,026)
S	7,179	1,480	7,113	1,011	7,039	1,204	6,852	1,823
	(1,480)	(7,113)	(1,011)	(7,039)	(1,204)	(6,852)	(1,823)	(7,349)

Table 2.3 Mean (± SEM, $n = 3$) trace element concentrations (mg kg^{-1}) of the experimental feeds.

Marker	Chromium				Yttrium			
Conc.	0.001%	0.01%	0.1%	1.0%	0.001%	0.01%	0.1%	1.0%
Ba	5.86	0.00	5.74	0.00	5.90	0.00	5.79	0.00
	(0.00)	(5.74)	(0.00)	(5.90)	(0.00)	(5.79)	(0.00)	(5.54)
Cd	0.53	0.00	0.38	0.07	0.40	0.01	0.38	0.00
	(0.00)	(0.38)	(0.07)	(0.40)	(0.01)	(0.38)	(0.00)	(0.27)
Co	1.29	0.00	2.43	0.45	5.21	6.31	2.09	0.45
	(0.00)	(2.43)	(0.45)	(5.21)	(6.31)	(2.09)	(0.45)	(1.42)
Cu	16.55	0.07	16.25	0.37	15.47	0.16	15.98	0.11
	(0.07)	(16.25)	(0.37)	(15.47)	(0.16)	(15.98)	(0.11)	(17.99)
Mn	41.40	0.03	35.90	0.01	38.35	0.01	38.53	0.32
	(0.03)	(35.90)	(0.01)	(38.35)	(0.01)	(38.53)	(0.32)	(42.84)
Se	0.25	0.03	1.72	0.52	0.78	0.30	1.44	0.63
	(0.03)	(1.72)	(0.52)	(0.78)	(0.30)	(1.44)	(0.63)	(0.83)
Si	41.08	0.44	38.03	0.74	40.41	0.09	37.96	1.09
	(0.44)	(38.03)	(0.74)	(40.41)	(0.09)	(37.96)	(1.09)	(42.44)
Y	25.17	0.54	4.56	0.09	0.74	0.01	0.61	0.00
	(0.54)	(4.56)	(0.09)	(0.74)	(0.01)	(0.61)	(0.00)	(6.31)
Zn	65.07	0.24	66.34	0.15	64.35	1.85	66.50	0.28
	(0.24)	(66.34)	(0.15)	(64.35)	(1.85)	(66.50)	(0.28)	(66.55)

2.2.1.2 Experimental system, fish and faecal collection

The experiment was conducted at the School of Aquaculture, University of Tasmania. One thousand four hundred and forty Atlantic salmon (*Salmo salar* L.) from Springfield Hatchery (Springfield, Tasmania) were allocated to 24 300-L tanks comprising the experimental system, held in a constant environment room. Photoperiod was held constant at 12-hour day length. Physically treated, bio-filtered, freshwater was supplied to each tank in a partial replacement system with a continuous replacement of approximately 10% per day from the municipal water supply. The system supplied filtered water to each tank at an average flow rate of 6 l min^{-1}. Water parameters (dissolved oxygen, oxygen saturation, chlorine, pH, ammonia, nitrate and nitrite) were monitored to ensure water quality remained within limits recommended for Atlantic salmon (Wedemeyer, 1996). Water temperature was recorded daily, and ranged from 14.2 – 16.0 °C with a mean of 15.0 ± 0.2 °C.

Prior to the start of the experiment, fish were anaesthetised (50 ml l^{-1}, benzocaine) and groups of five fish were weighed to the nearest 0.1 g and distributed to 24 tanks, until a stocking rate of 60 fish per tank was attained. Adjustments were made to balance the total biomass of fish per tank, rather than the number of fish per tank to reduce the likelihood of significant differences in faecal output. The mean total weight of fish for all tanks was 2229 g (± 12.3 g), and ranged from 2205 g to 2252 g. The mean number of fish was 60 (± 4.3 fish per tank), and ranged from 54 to 69 fish per tank. Each of the eight feeds was assigned to three tanks, one in each of three rows within the testing facility. The fish were acclimatised to the tanks for seven days prior to the start of the experiment.

Fish faeces were collected using a simple modification of the Guelph design (Cho & Slinger, 1979), as described in Carter and Hauler (2000). The modification included the use of a widened portion on the outflow pipe, to facilitate the settlement of faecal material, above a collection pot. The attachment enabled a 70-ml sterile container to be put in place for each collection period (Fig. 2.1). Fish were switched from a commercial feed to the experimental feed and fed 1% BW in the morning and in the evening, for four days prior to faecal sampling. Faecal collections started one hour after the evening feeding, and lasted 18 h, and were collected over four days. After this initial sampling, the fish were pooled and redistributed to the tanks and adjusted by total biomass as before. The feed treatments were reassigned to the tanks, and the feeding and collection process repeated. Faecal samples were frozen immediately after collection and stored at -4 ° C. All faecal samples were then freeze-dried together at -10 to -12 °C and -100 to -110 kPa until all samples reached a constant weight.

2.2.2 Experiment 2 – Sampling time periods

The marked feed containing 0.1% yttrium oxide from the first experiment was used to observe the effect of faecal collection time on mineral and trace element determinations. The experimental system and system parameters remained the same as in experiment 1.

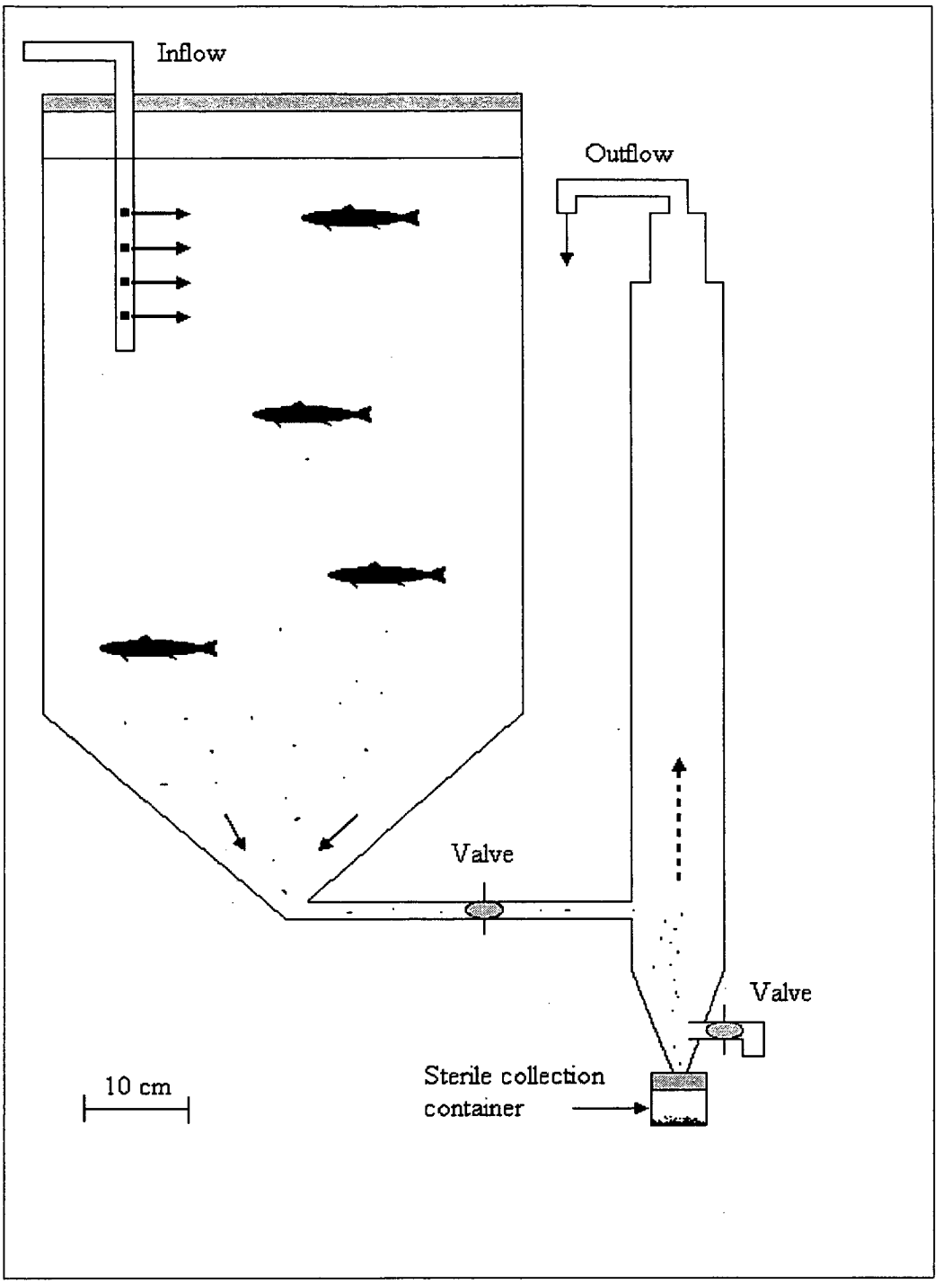

Figure 2.1 The experimental tank with attached faecal collection unit. This modification of the Guelph collection system includes a connector that allows the removal and replacement of a sterile sample collection container (70 ml). This permitted the recovery of the water in the container immediately surrounding the faecal sample.

One hundred and eighty Atlantic salmon were weighed in groups of five fish and randomly distributed to each of six tanks, until a total of 30 fish per tank was attained. The mean total biomass of fish for all tanks was 2493 g (± 47.7 g), and ranged from 2432 g to 2576 g. The mean weight of individual fish was 83.1 g (± 1.5 g), as determined from groups of five fish. The fish were acclimatised to the tanks for seven days prior to collection of faeces.

Fish were switched from a commercial feed, to the marked feed, and were fed to satiation once per day at 0900, corresponding to the start of the light cycle, for four days prior to faecal sampling. Faeces were collected over a 24 h period according to three different sampling regimes consisting of either four 6 h, two 12 h or one 24 h collections. The treatments, faecal collection timing, were randomly allocated to one tank in each of the two rows for collections taken over a period of three days. Samples collected at 6 and 12 h intervals were immediately frozen, and a new sterile container placed on the settlement collection units for the next collection interval. The samples were processed as described previously.

2.2.3 Analytical procedures

2.2.3.1 Sample decomposition and analyses

Freeze-dried faecal samples were weighed to the nearest 0.0001 g and samples of each experimental feed were subjected to wet-decomposition at 100 °C with 10 ml of nitric acid (Aristar Grade, 16 M HNO_3), left to cool, then 10 ml of hydrogen peroxide (30% w/v) was added and the samples returned to 100 °C. After decomposition the

samples were made up to a volume of 50 ml with purified, de-ionised water. The samples were decomposed by personnel at the Central Science Laboratory (University of Tasmania, Hobart, Tasmania, Australia).

The samples were analysed for the external markers, chromium oxide and yttrium oxide, and a full range of minerals and trace elements using inductively coupled plasma optical emission spectrophotometry (Thermo Jarrell-Ash IRIS Axial ICP-OES) at the Central Science Laboratory (University of Tasmania, Hobart, Tasmania, Australia). Samples were diluted a further 20-fold to improve the determination of highly concentrated minerals, such as phosphorus and calcium. Any element found in concentrations below ICP-OES detection limits was not reported. Blank samples, containing only the decomposition acid, were included to measure the matrix effects of decomposition, which were subtracted from every element in each sample. Mineral and trace element quality control standard samples were used to assess the accuracy of the ICP-OES analysis, and were within 5% of known concentrations for all elements.

2.2.3.2 Chromium analysis using the optical density method

Additional chromium determinations were conducted using a modification of the optical density method described by Furukawa and Tsukahara (1966). Approximately 1.0 - 2.0 g of feed and 0.50 - 1.0 g of the remaining faecal material from the samples obtained in experiment 1, were weighed to the nearest 0.0001 g into decomposition tubes. The samples were decomposed with 4.0 ml concentrated nitric acid (AnalaR

grade, 16 M HNO_3), initially at room temperature over 24 h, then heated to 150 °C for at least an hour. Additional nitric acid was added to maintain at least 4.0 ml of liquid in each sample. The samples were then allowed to cool to room temperature, and 5.0 ml of concentrated perchloric acid (AnalaR grade, 70% $HClO_4$) was added to each sample and then placed in a heating block at 220 °C for 30 min. Additional perchloric acid was added to samples to maintain at least 2.0 to 3.0 ml of liquid in each sample. The samples were then heated to 245 °C until all samples changed to an orange/yellow colour, and then the samples were decomposed for an additional 30 min, then removed from the heat. After the samples were allowed to cool, the walls of the decomposition tubes were washed with distilled water and transferred to either 50 or 500 ml volumetric flasks, and made up with distilled water. The determination of which size volumetric flask to use was determined by a visual estimate of the concentration of the colour, optical density, in each sample at the end of the digestion. Samples were initially diluted to 50 ml, and were diluted to 500 ml as required for analysis. The samples were read on a spectrophotometer at 346.5 nm for 3 seconds, after the machine was zeroed with blank samples that contained only the decomposition acids.

2.2.3.3 Acid insoluble ash

The feed marked with yttrium oxide at 0.1% and faecal samples collected from that feed in the first experiment were analysed for acid insoluble ash (AIA) using the method described by Atkinson et al. (1984), and ADC calculated from these values.

ADC were derived for each mineral using the mineral and trace element concentrations determined for those samples by ICP-OES.

2.2.3.4 Apparent digestibility coefficients

Apparent digestibility coefficients (ADC) were determined for each mineral and trace element that was present in reportable concentrations. The ADC were determined according to the following equation (Maynard & Loosli, 1969):

$$ADC(\%) = 100 - \left[\frac{[M]feed \times [N]faeces}{[M]faeces \times [N]feed}\right] \times 100 \qquad [2.1]$$

where [*M*] was the concentration (mg kg^{-1}), of the digestibility marker, and [*N*] the concentration of the nutrient (mg kg^{-1}). The mean chromium concentrations, determined by the optical density method, and the mean AIA concentrations were used to estimate ADC using the same formula.

2.2.4 Statistical analyses

All statistical analyses were performed using SPSS v. 10.0 software (SPSS, 2000) following the statistical methods described in Zar (1984) and Underwood (1981). For statistical analysis of ADC the experimental unit was the tank. All ADC data were

tested for normality (Shapiro-Wilk), and, where applicable, ADC (%) data was arcsine transformed with the following equation (Zar, 1984):

$$ADC' = \arcsin\sqrt{ADC} \qquad [2.2]$$

Data was analysed using a one-way ANOVA to compare the difference between means of the mineral and trace element ADC resulting from each marker, marker concentration or sampling periods. Tukey's honestly significant difference test (Tukey's HSD) was used for multiple comparison of means for all data. Significance for all statistical tests was accepted at probability levels of 0.05 or less. The Y_2O_3 0.01% feed samples contained no detectable concentrations of cadmium and the ADC for cadmium for this feed were not calculated or included in any statistical calculations.

Some calculated ADC data were negative, resulting from greater concentrations of an element in the faeces than the feed, and it was not possible to arcsine transform these values. Statistical analysis of faecal collection timing was conducted on the mean values derived from all samples collected over a 24 h period collection when comparing sampling time periods, and on the mean value of sample numbers when comparing multiple samples taken within a 24 h period.

2.3 Results

2.3.1 Experiment 1: Markers

2.3.1.1 Recovery of external markers

The percentage of external markers recovered from the feed, as determined by ICP-OES, ranged from 80.1% to 96.0% for yttrium oxide and 0.1% to 10.6% for chromium oxide (Table 2.4). The 0.1% yttrium oxide feed had the highest recovery rate, and, along with the 1.0% feed, had significantly higher recovery rates than the other yttrium marked feeds. The considerably lower recovery rates for chromium oxide and the presence of a green precipitate, the colour of the chromium oxide, in the decomposition containers indicated a failure of the nitric acid to completely decompose chromium oxide. For this reason the chromium content of the feeds and faeces were reanalysed using the optical density method (see above), which decomposes samples with concentrated perchloric acid.

The optical density method was effective for determining chromium content in the feeds with 1.0% and 0.1% inclusion levels, however, the recovery rates were greater than 140% and 570% for the 0.01% and 0.001% respectively, with the lowest inclusion rate significantly different from the others (Table 2.4). The chromium inclusion of 1.0% was the least variable, as measured by the standard error of the mean for the recovery rate. Since the measurements for the two lowest inclusion levels were only 1 - 5 times greater than the detection limit of this analytical procedure, these were not included in statistical analyses. This method produced chromium content measurements from the faecal samples that were consistent with

Table 2.4 Recovery of external markers from the experimental feeds

Parameter	Marker Concentration				F value	*P*
	1.0%	0.1%	0.01%	0.001%	(*df*=3)	
Expected Y[1]	7875.0	787.5	78.75	7.87		
Y_2O_3 ICP-OES	7285.1^{c} (6105.7)	755.8^{b} (17.4)	65.01^{a} (0.1)	6.31^{a} (0.0)	1202.40	<0.001
Recovery (%)	92.5^{b} (1.0)	96.0^{b} (0.3)	82.6^{a} (0.2)	80.1^{a} (0.5)	73.04	<0.001
Expected Cr[1]	6840.0	684.0	68.4	6.8		
Cr_2O_3 ICP-OES	20.1^{b} (6.84)	23.1^{ab} (11.99)	14.2^{ab} (5.64)	7.0^{a} (0.96)	5.57	0.019
Recovery (%)	0.1^{b} (0.0)	0.2^{b} (0.0)	2.1^{b} (0.0)	10.6^{a} (0.7)	78.83	<0.001
Cr_2O_3 Optical	7311.53^{c} (593.19)	662.37^{b} (157.93)	98.05^{a} (16.64)	39.04^{a} (10.35)	692.29	<0.001
Recovery (%)	106.89^{a} (8.67)	96.84^{a} (23.09)	143.34^{a} (24.33)	570.82^{b} (151.30)	32.92	<0.001

Means (± SEM, $n = 6$) with the same superscript were not significantly different (Tukey's HSD).

[1]Expected yttrium and chromium concentrations (mg kg^{-1}) based on the proportion of each element from the formulated inclusion concentration of each marker for Y_2O_3 and Cr_2O_3 respectively.

the inclusion levels, with the higher inclusion rates having chromium contents higher than lower inclusion rates.

2.3.1.2 ADC from external and internal markers

The mean ADC derived from the yttrium oxide inclusion levels for minerals and trace elements (Table 2.5 and 2.6) showed significant differences for all minerals except potassium and all trace elements that had reportable concentrations. The experimental feed containing 0.1% Y_2O_3 feed displayed consistently lower mean ADC for minerals and trace elements; the only exception was chromium, where the mean ADC was highest (65.7 ± 24.5%). The higher recovery of the yttrium oxide from the feed can account for a degree of these differences, as could differences in the rate of passage of this marker though thee gastrointestinal tract of the fish. Those minerals and trace elements with the greatest inclusion concentrations tended to have lower significant differences in mean ADC.

It was possible to calculate ADC from feed and faeces marked with chromium oxide using the optical density method of analysis but, the inclusion levels of 0.01% and 0.001% were much greater than expected and were not reported. There were significant differences between these two marker concentrations for almost all elements (Tables 2.7 and 2.8) except boron, potassium, yttrium and zinc. The ADC calculated for iron, potassium, sodium, phosphorus, sulphur, barium, cobalt, copper, selenium, silicon and zinc were comparable to those determined with the yttrium marker.

Table 2.5 The effect of the yttrium oxide concentration on mean (± SEM, $n = 6$) mineral ADC (%)

Element	Yttrium concentration				F value	*P*
	1.0%	0.1%	0.01%	0.001%	(*df*=3)	
Ca	-25.73[a] (4.03)	-29.18[a] (5.12)	-26.22[a] (2.97)	-6.49[b] (13.02)	7.90	0.001
Fe	12.18[b] (1.88)	-9.48[a] (6.80)	12.40[b] (0.36)	30.91[c] (3.67)	34.42	<0.001
K[1]	99.78 (0.00)	99.76 (0.00)	99.81 (0.00)	99.80 (0.00)	0.59	ns
Mg	32.44[b] (1.02)	17.19[a] (1.35)	22.46[a] (0.98)	32.15[b] (3.55)	16.97	<0.001
Na	92.90[ab] (0.27)	91.03[a] (0.20)	92.87[ab] (0.33)	94.53[b] (0.12)	5.29	0.008
P	53.74[bc] (0.51)	46.91[a] (0.26)	49.33[ab] (0.57)	55.80[c] (2.07)	11.04	<0.001
S	79.93[b] (0.17)	76.49[a] (0.19)	79.42[b] (0.37)	82.16[b] (0.30)	13.23	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

[1]The data for this element was arcsine transformed prior to statistical analysis.

Table 2.6 The effect of the yttrium oxide concentration on mean (± SEM, $n = 6$) trace element ADC (%)

Element	Yttrium concentration				F value	*P*
	1.0%	0.1%	0.01%	0.001%	(*df*=3)	
Ba	-0.24^{ab} (7.68)	-13.42^{a} (5.12)	-8.60^{ab} (2.14)	3.49^{c} (9.90)	4.70	0.012
Cd[2]	-2.15^{b} (13.36)	-19.60^{a} (5.42)		-15.52^{a} (30.14)	36.17	<0.001
Co	65.40^{b} (8.03)	26.19^{a} (19.390	69.93^{b} (9.66)	56.16^{b} (2.75)	16.94	<0.001
Cr[1]	38.09^{a} (1.02)	65.56^{b} (24.51)	32.14^{a} (5.71)	37.08^{ab} (28.48)	6.53	0.003
Cu[1]	40.91^{a} (3.16)	38.79^{a} (6.21)	53.80^{b} (5.81)	56.24^{b} (0.84)	8.96	0.001
Mn	9.65^{b} (1.66)	-11.21^{a} (0.34)	-2.68^{a} (6.71)	18.17^{b} (5.65)	35.44	<0.001
Se	-61.43^{c} (785.61)	-1383.5^{a} (12431.0)	-622.88^{b} (14214.7)	-108.56^{c} (447.68)	29.88	<0.001
Si	-12.45^{a} (4.43)	-10.48^{a} (8.79)	5.60^{ab} (6.780	17.78^{b} (15.47)	10.78	<0.001
Zn[1]	49.73^{b} (1.08)	37.53^{ab} (0.60)	23.76^{a} (43.96)	42.32^{ab} (28.95)	3.26	0.044

Means with the same superscript were not significantly different (Tukey's HSD).

[1]The data for this element was arcsine transformed prior to statistical analysis.

[2]Cadmium values for the 0.01% feed were removed from statistical calculations due to extreme outliers in the feed samples used to calculate the ADC.

Table 2.7 The effect of chromium concentrations, derived from the optical density method, on mean (± SEM, $n = 6$) mineral ADC (%)

Element	Marker concentration		F value	*P*
	1.0%	0.1%	(*df*=3)	
Ca	-7.07 (5.45)	-59.90 (7.87)	183.42	<0.001
Fe	8.80 (10.23)	-21.55 (5.56)	40.94	<0.001
K	99.79 (0.05)	99.60 (0.20)	3.28	ns
Mg	16.69 (13.77)	-19.40 (12.67)	22.35	.001
Na	92.99 (2.78)	89.16 (2.07)	7.30	.022
P	50.03 (3.69)	27.10 (5.27)	75.87	<0.001
S	76.11 (2.95)	66.66 (2.30)	38.25	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

Table 2.8 The effect of chromium concentrations, derived from the optical density method, on mean (± SEM, $n = 6$) trace element ADC (%)

Element	Marker concentration		F value	*P*
	1.0%	0.1%	(*df*=3)	
B	-274.53 (103.14)	-201.60 (119.93)	1.27	ns
Ba	-14.08 (18.05)	-46.07 (10.53)	14.07	0.004
Cd	12.07 (7.14)	-19.52 (6.62)	62.97	<0.001
Co	65.19 (8.22)	80.49 (3.86)	17.12	0.002
Cu	42.71 (8.39)	5.52 (35.61)	6.20	0.032
Mn	-1.61 (4.45)	-48.94 (24.21)	22.22	0.001
Se	-67.17 (87.54)	-523.11 (275.39)	14.93	0.003
Si	14.82 (27.32)	-21.26 (25.95)	5.49	0.041
Y	-793.18 (1443.13)	-2295.79 (5212.26)	0.46	ns
Zn	22.63 (34.99)	8.08 (10.91)	0.94	ns

Means with the same superscript were not significantly different (Tukey's HSD).

There was sufficient faecal material available from the Y_2O_3 0.1% feed and its corresponding faecal samples to measure acid insoluble ash and calculate ADC for comparison with those calculated by yttrium and the chromium oxide 1.0% (Table 2.9 and 2.10). The feeds and faecal samples marked with 0.1% of Y_2O_3 contained 2189 ±671 mg kg^{-1} and 5655 ±853 mg kg^{-1} of acid insoluble ash respectively. These mean AIA values and the elemental concentrations from the ICP-OES for the Y_2O_3 0.1% feed and faeces were used to calculate ADC with AIA. Chromium ADC of 39.4 ± 28.9% and 38.1 ±3.5% were calculated with AIA and yttrium oxide respectively. ADC calculated with AIA were significantly lower from those calculated with Y_2O_3 1.0% and Cr_2O_3 1.0%. Y_2O_3 1.0% provided ADC similar to those derived from chromium 1.0% in most cases, except calcium, magnesium, boron and manganese. The significant differences in the boron ADC calculated for chromium were the product of relatively low concentrations in that feed.

Regardless of marker or concentration, elements with the greatest concentrations showed the least amount of differences in ADC and the lowest standard error of means. Potassium displayed an unusually consistent ADC. Calcium provided consistent negative ADC when calculated with the yttrium marker. Sodium possessed ADC of 90% or better, but was more variable than potassium. Elements such as selenium, molybdenum, nickel, lead, tin, boron and arsenic were often found in concentrations less than the values provided by blank samples. None of these values were reported or used to determine ADC.

Table 2.9 A comparison of mean (± SEM, $n = 6$) mineral ADC (%) calculated using an internal marker (AIA) and external markers (Cr_2O_3 and Y_2O_3)

Element	Marker			F value	*P*
	AIA	Cr_2O_3 (1.0%)	Y_2O_3 (1.0%)	(*df*=1)	
Ca	-127.64[a] (10.96)	-7.08[b] (5.43)	-25.73[c] (6.94)	418.07	<0.001
Fe	-93.23[a] (19.62)	8.80[b] (10.20)	12.18[b] (4.76)	131.95	<0.001
K	99.56[a] (0.07)	99.77[b] (0.05)	99.78[b] (0.04)	30.61	<0.001
Mg	-46.16[a] (9.52)	16.67[b] (13.75)	32.43[c] (3.48)	128.28	<0.001
Na	84.15[a] (3.23)	92.98[b] (2.77)	92.92[b] (1.81)	24.73	<0.001
P	6.39[a] (2.73)	50.03[b] (3.71)	53.73[b] (2.47)	560.19	<0.001
S	58.46[a] (3.91)	76.12[b] (2.95)	79.95[b] (1.43)	99.45	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

Table 2.10 Comparison of mean (± SEM, $n = 6$) trace element ADC (%) calculated using an internal marker (AIA) and external markers (Cr_2O_3 and Y_2O_3)

Element	Marker			F value	*P*
	AIA	Cr_2O_3 (1.0%)	Y_2O_3 (1.0%)	(*df*=2)	
B	-99.81[b] (121.16)	-274.55[a] (103.14)	-0.23[b] (9.59)	12.636	<0.001
Ba	-99.89[a] (12.88)	-14.08[b] (18.04)	-15.50[b] (9.54)	91.118	<0.001
Cd	-111.39[a] (21.14)	12.05[b] (7.15)	36.30[c] (7.91)	206.900	<0.001
Co	-30.45[a] (32.63)	65.18[b] (8.21)	65.42[b] (9.82)	45.230	<0.001
Cr[1]	39.44 (28.98)	n/a	38.10 (3.49)	0.012	ns
Cu	-8.10[a] (18.48)	42.72[b] (8.38)	40.92[b] (6.15)	34.725	<0.001
Mn	-96.25[a] (6.21)	-1.62[b] (4.42)	9.65[c] (4.46)	885.897	<0.001
Se	-2514.36[a] (793.87)	-67.17[b] (87.54)	-61.43[b] (97.10)	54.461	<0.001
Si	-94.84[a] (20.17)	14.82[b] (27.33)	-12.43[b] (7.31)	57.701	<0.001
Y[1]	-76.48 (5.15)	-793.18 (1443.15)	n/a	2.029	ns
Zn	-10.38[a] (8.38)	22.62[b] (34.98)	47.68[b] (3.59)	15.069	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

[1] ADC cannot be calculated for a marker itself, therefore, a one-way analysis of variance ($df = 1$) was conducted on the remaining markers.

2.3.2 Experiment 2: Effect of sampling period

Only potassium, sodium, sulphur and aluminium showed significant differences between ADC calculated from faecal samples collected for four 6 h, two 12 h or entire 24 h time periods (Tables 2.11 and 2.12). However, most elements displayed significantly different ADC when comparing samples taken at 6 h intervals (Tables 2.13 and 2.14) and 12 h intervals (Tables 2.15 and 2.16). Magnesium and manganese stand out as elements where sampling time period had the greatest effect on ADC. Yttrium concentrations also varied over 24 h sampling periods (Figure 2.2). General trends are apparent when comparing the differences in sampling period for several elements with significantly different ADC. Minerals and trace elements show either subtle or marked decreases in ADC over time, such as cadmium and phosphorus (Figure 2.3), and magnesium and manganese respectively (Figure 2.4). The ratio of marker to element concentration in these faecal samples varied significantly with sampling time period for magnesium and manganese (Figure 2.5). Iron stands out as an exception displaying a marked increase in ADC depending on sampling period (Figure 2.6). The ratios of marker to iron in the initial 6 h samples (0.61 ± 0.03) were significantly greater ($F = 20.05$, $df = 4$, $P < 0.001$) than the remaining 6 h samples or 24 h samples (0.45 ± 0.05). There were significant differences in the ratios of other elements to yttrium, but there were no trends as apparent in the previous examples.

Table 2.11 The effect of collecting faecal samples over 24 h in 6-, 12-, and 24-hour periods on mean (± SEM, $n = 6$) mineral ADC (%)

Element	Sampling period[1]			F value	*P*
	6-hour	12-hour	24-hour	(*df*=2)	
Ca	22.48 (1.18)	24.03 (1.97)	24.54 (5.36)	0.29	ns
Fe	-9.18 (7.51)	-8.88 (6.60)	-9.18 (8.56)	0.00	ns
K	99.61^{b} (0.00)	99.50^{ab} (0.00)	98.95^{a} (0.17)	3.08	0.056
Mg	42.49 (4.64)	38.05 (9.65)	39.38 (0.94)	0.45	ns
Na	87.16^{b} (0.06)	84.07^{ab} (0.79)	82.81^{a} (1.02)	7.27	0.002
P	61.40 (0.61)	59.05 (0.95)	59.77) (1.34)	0.95	ns
S	81.80^{b} (0.10)	79.21^{ab} (0.57)	77.68^{a} (1.25)	6.17	0.005

Means with the same superscript were not significantly different (Tukey's HSD).

[1]Statistical analysis was performed on the mean of 24 hours of sampling.

Table 2.12 The effect of collecting faecal samples over 24 h in 6-, 12-, and 24-hour periods on mean (± SEM, $n = 6$) trace elements ADC (%)

Element	Sampling period[1]			F value	*P*
	6-hour	12-hour	24-hour	(*df*=2)	
Al	-422.07[a] (1330.52)	-267.10[ab] (930.32)	-177.44[b] (2902.38)	4.25	0.021
B	-120.98 (353.54)	-133.55 (682.19)	-87.01 (909.21)	0.30	ns
Ba	-1.07 (2.66)	-0.12 (3.20)	3.34 (3.68)	0.50	ns
Cd	-15.98 (4.38)	-19.84 (7.32)	-20.70 (8.42)	0.50	ns
Co	33.38 (4.68)	25.56 (4.49)	25.14 (22.58)	1.67	ns
Cu	49.04 (0.44)	46.08 (4.61)	43.38 (2.86)	1.90	ns
Mn	8.51 (3.03)	7.15 (6.07)	8.39 (3.96)	0.06	ns
Mo	-8.39 (174.58)	-34.85 (236.77)	-22.36 (247.58)	0.42	ns
Si	-50.56 (24.72)	-32.09 (56.17)	-22.10 (205.21)	1.88	ns
V	-51.69 (766.33)	-112.37 (1053.40)	-8.20 (3264.92)	0.78	ns
Zn	43.38 (2.78)	47.89 (11.02)	43.57 (2.15)	0.55	ns

Means with the same superscript were not significantly different (Tukey's HSD).

[1]Statistical analysis was performed on the mean of 24 hours of sampling.

Table 2.13 Mean (± SEM, $n = 6$) mineral ADC (%) calculated from consecutive 6-hour faecal sampling periods

Element	Sampling period (h)				F value	*P*
	0-6	6-12	12-18	18-24	(*df*=3)	
Ca	24.15^{b} (2.90)	28.71^{b} (1.99)	23.37^{b} (1.50)	13.72^{a} (3.41)	8.09	0.001
Fe	-37.46^{a} (2.80)	-6.45^{b} (7.95)	4.54^{b} (10.65)	2.59^{b} (3.40)	30.56	<0.001
K	99.48^{a} (0.00)	99.57^{ab} (0.00)	99.70^{b} (0.00)	99.71^{b} (0.00)	3.67	0.029
Mg	60.88^{d} (1.60)	49.47^{c} (1.04)	36.34^{b} (1.83)	23.29^{a} (1.52)	87.78	<0.001
Na	85.96 (0.22)	86.58 (0.13)	87.88 (0.25)	88.20 (0.25)	2.64	ns
P	63.43^{b} (1.44)	65.14^{b} (0.46)	62.98^{b} (0.97)	54.09^{a} (0.88)	13.25	<0.001
S	79.76^{a} (0.19)	83.36^{b} (0.11)	83.01^{b} (0.28)	81.14^{ab} (0.35)	6.07	0.004

Means with the same superscript were not significantly different (Tukey's HSD).

Table 2.14 Mean (± SEM, $n = 6$) trace element ADC (%) calculated from consecutive 6-hour faecal sampling periods

Element	Sampling period (h)				F value	*P*
	0-6	6-12	12-18	18-24	(*df*=3)	
Al	-255.57^{b} (946.72)	-445.65^{ab} (4205.55)	-654.09^{a} (7881.16)	-332.98^{ab} (2443.32)	3.87	0.025
B	-73.70^{ab} (353.97)	-131.83^{ab} (295.14)	-233.82^{a} (3501.59)	-44.55^{b} (261.52)	3.16	0.047
Ba	2.29 (4.03)	2.35 (21.43)	1.60 (4.42)	-10.51 (7.23)	2.14	ns
Cd	-5.02^{b} (3.99)	-5.38^{b} (7.09)	-20.79^{a} (7.24)	-32.71^{a} (8.71)	13.23	<0.001
Co	22.74^{a} (13.77)	46.65^{b} (11.67)	35.48^{ab} (6.57)	28.65^{ab} (22.52)	3.85	0.025
Cu	45.78^{a} (1.85)	53.04^{b} (1.51)	48.89^{ab} (0.85)	48.46^{ab} (1.18)	3.33	0.040
Mn	20.72^{c} (3.45)	14.57^{bc} (3.62)	4.06^{ab} (6.24)	-5.31^{a} (2.69)	16.52	<0.001
Mo	12.13 (116.22)	-48.30 (1592.79)	5.07 (877.21)	-2.48 (403.08)	0.49	ns
Si	-53.23 (90.10)	-48.66 (109.75)	-54.43 (124.02)	-45.92 (126.21)	0.07	ns
V	-51.30 (5060.72)	-42.99 (2557.33)	-100.95 (3642.45)	-11.51 (2428.32)	0.20	ns
Zn	49.15^{b} (6.11)	48.64^{b} (3.02)	43.66^{ab} (6.26)	32.09^{a} (17.00)	3.86	0.025

Means with the same superscript were not significantly different (Tukey's HSD).

Table 2.15 Mean (± SEM, $n = 6$) mineral ADC (%) calculated from 12-hour sampling periods

Element	Sampling period (h)		F value	*P*
	0-12	12-24	(*df*=3)	
Ca	26.88 (2.10)	21.60 (4.73)	1.86	ns
Fe	-16.05 (4.72)	-2.76 (14.35)	4.23	ns
K	99.46 (0.00)	99.51 (0.00)	0.39	ns
Mg	53.55 (0.29)	24.79 (3.81)	87.00	<0.001
Na	83.13 (1.65)	84.93 (1.64)	0.47	ns
P	62.14 (0.99)	56.40 (1.54)	6.17	0.030
S	79.33 (1.16)	79.09 (1.29)	0.01	ns

Table 2.16 Mean (± SEM, $n = 6$) trace element ADC (%) calculated from 12-hour sampling periods

Element	Sampling period (h)		F value	*P*
	0-12	12-24	(*df*=3)	
Al	-215.17 (3142.08)	-311.61 (853.45)	1.27	ns
B	-132.32 (3116.02)	-134.61 (307.93)	0.01	ns
Ba	7.00 (0.82)	-6.22 (4.56)	14.33	0.003
Cd	-9.23 (8.03)	-28.93 (6.51)	13.37	0.004
Co	25.77 (11.04)	25.38 (8.77)	0.01	ns
Cu	50.84 (10.91)	42.00 (6.33)	2.34	ns
Mn	19.02 (0.59)	-3.03 (3.45)	53.18	<0.001
Mo	-30.06 (408.40)	-38.95 (584.66)	0.03	ns
Si	-30.17 (138.92)	-33.73 (108.91)	0.02	ns
V	-68.37 (1874.09)	-150.08 (2317.17)	0.77	ns
Zn	47.09 (2.91)	48.57 (38.76)	0.02	ns

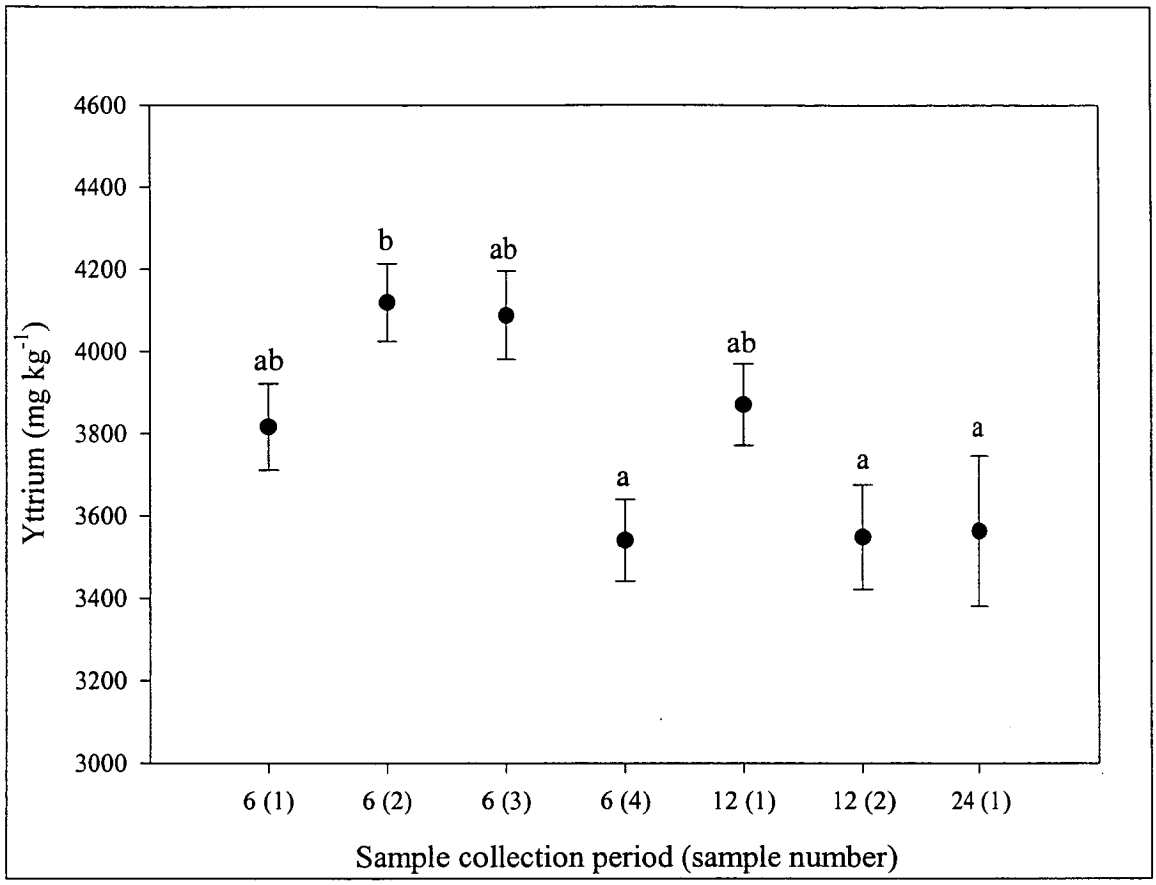

Figure 2.2 Mean (±SEM, $n = 6$) yttrium concentrations in faecal samples collected at differing lengths of time (6 h, 12 h and 24 h) over a 24-h period of collection. Sample collection period means with the same superscript were not significantly different (Tukey's HSD).

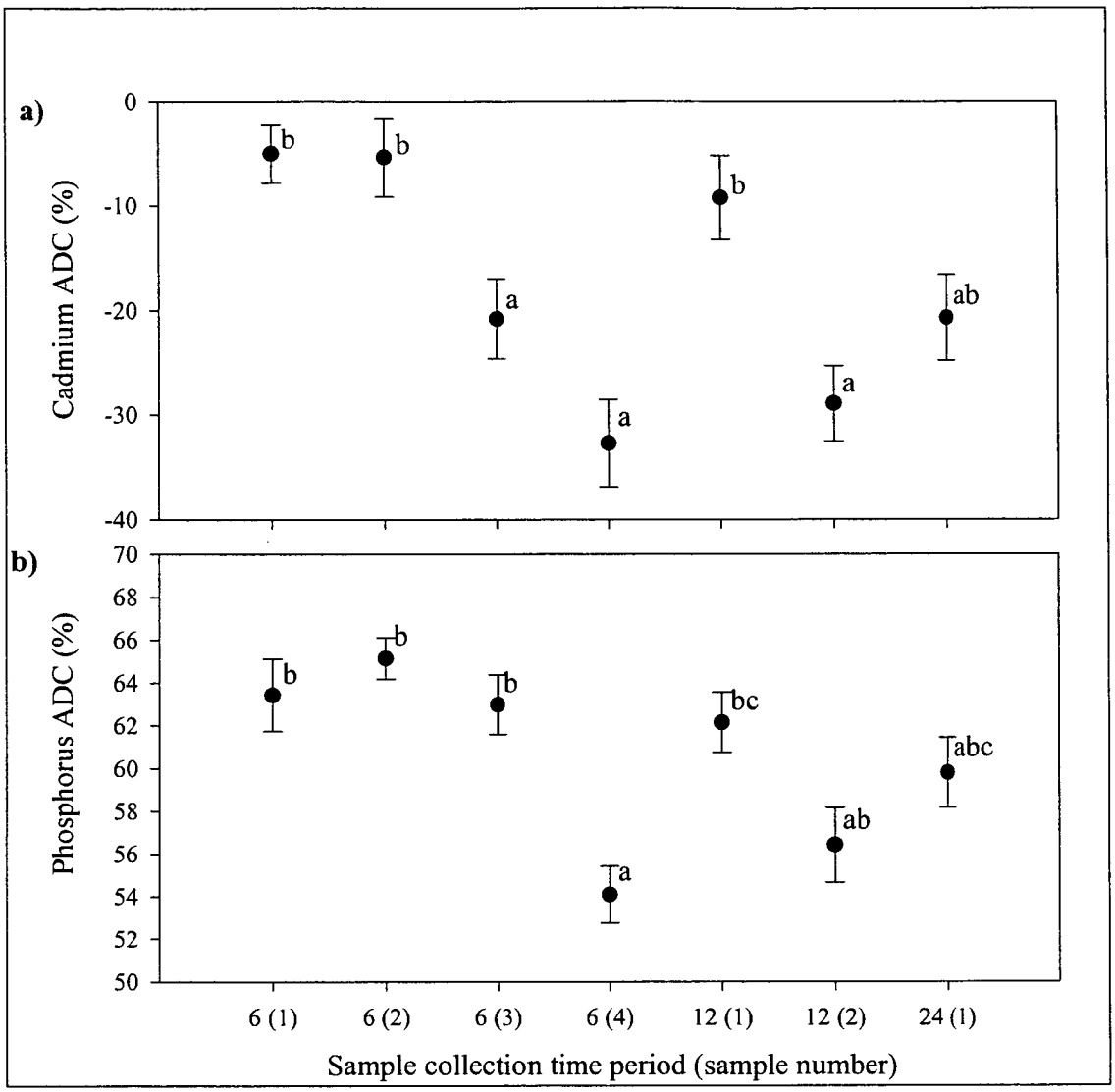

Figure 2.3 Mean (± SEM, $n = 6$) a) cadmium and b) phosphorus ADC (%) calculated from faecal samples collected at differing lengths of time (6 h, 12 h and 24 h) over a 24-h period of collection. Sample collection period means with the same superscript were not significantly different (Tukey's HSD).

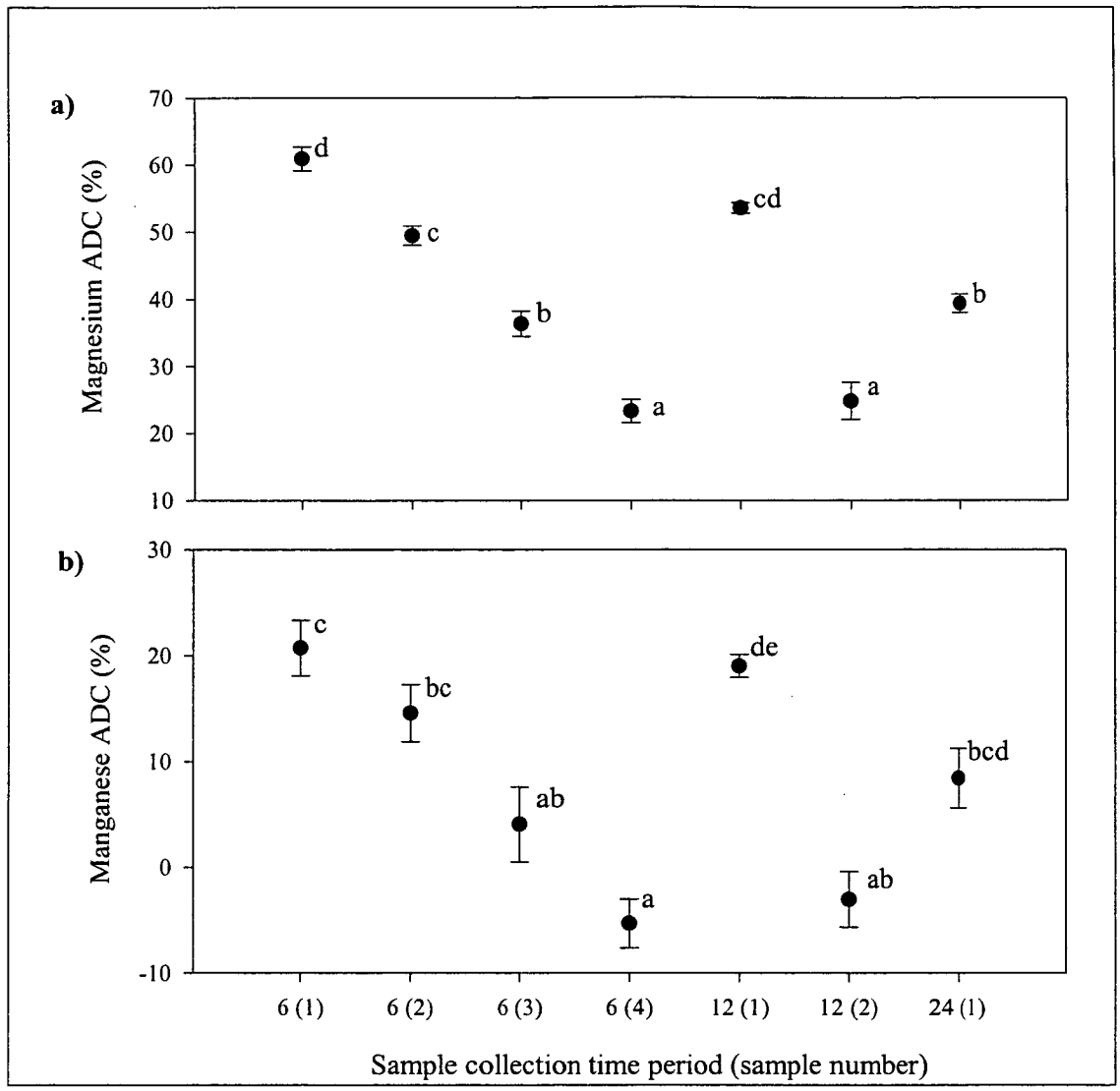

Figure 2.4 Mean (± SEM, $n = 6$) a) magnesium and b) manganese ADC (%) calculated from faecal samples collected at differing lengths of time (6 h, 12 h and 24 h) over a 24-h period of collection. Sample collection period means with the same superscript were not significantly different (Tukey's HSD).

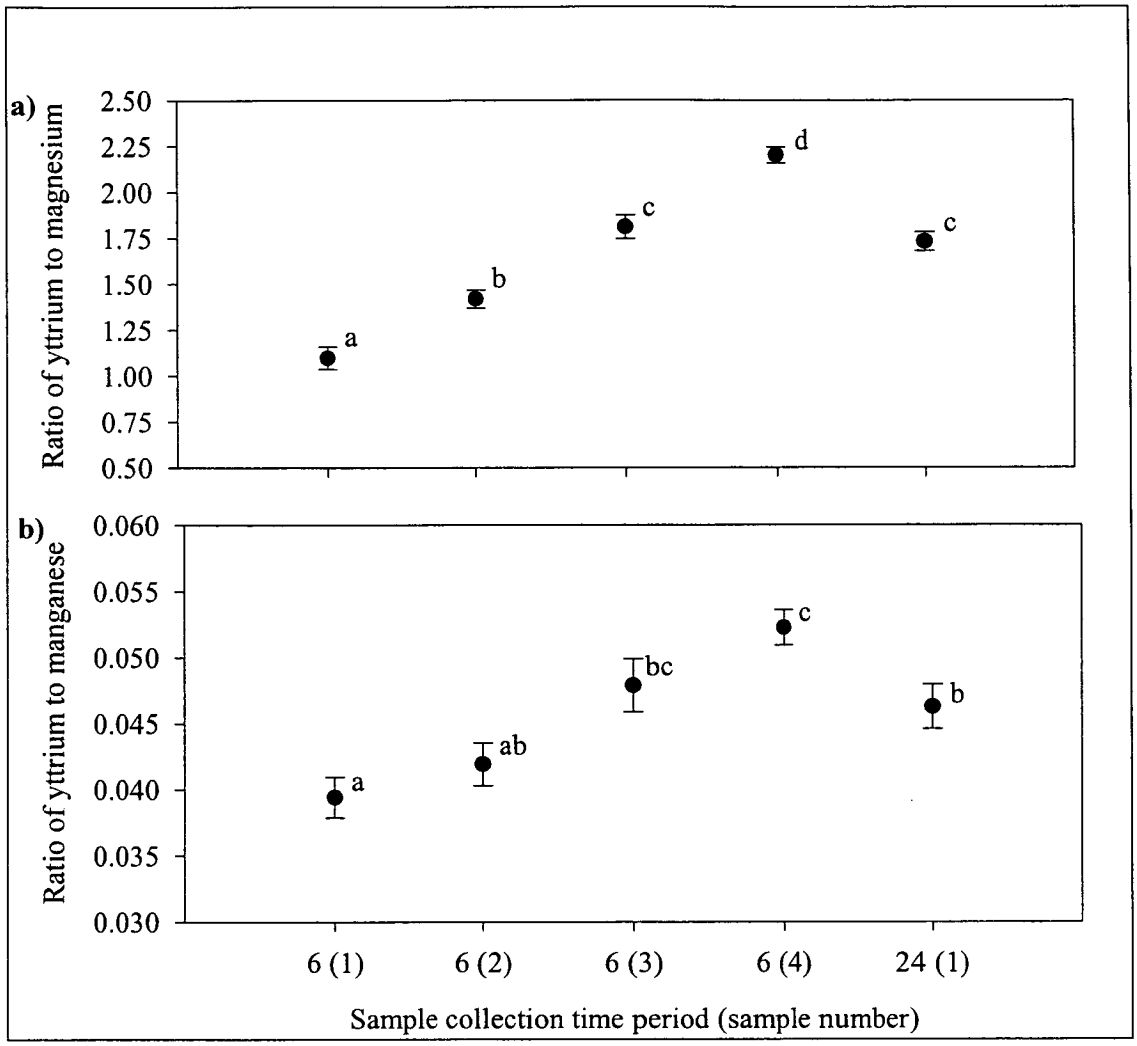

Figure 2.5 The mean (± SEM, $n = 6$) ratios of the concentration of yttrium to the concentrations of a) magnesium and b) manganese from faecal samples collected from consecutive 6-h samples or 24-h samples. Sample collection period means with the same superscript were not significantly different (Tukey's HSD).

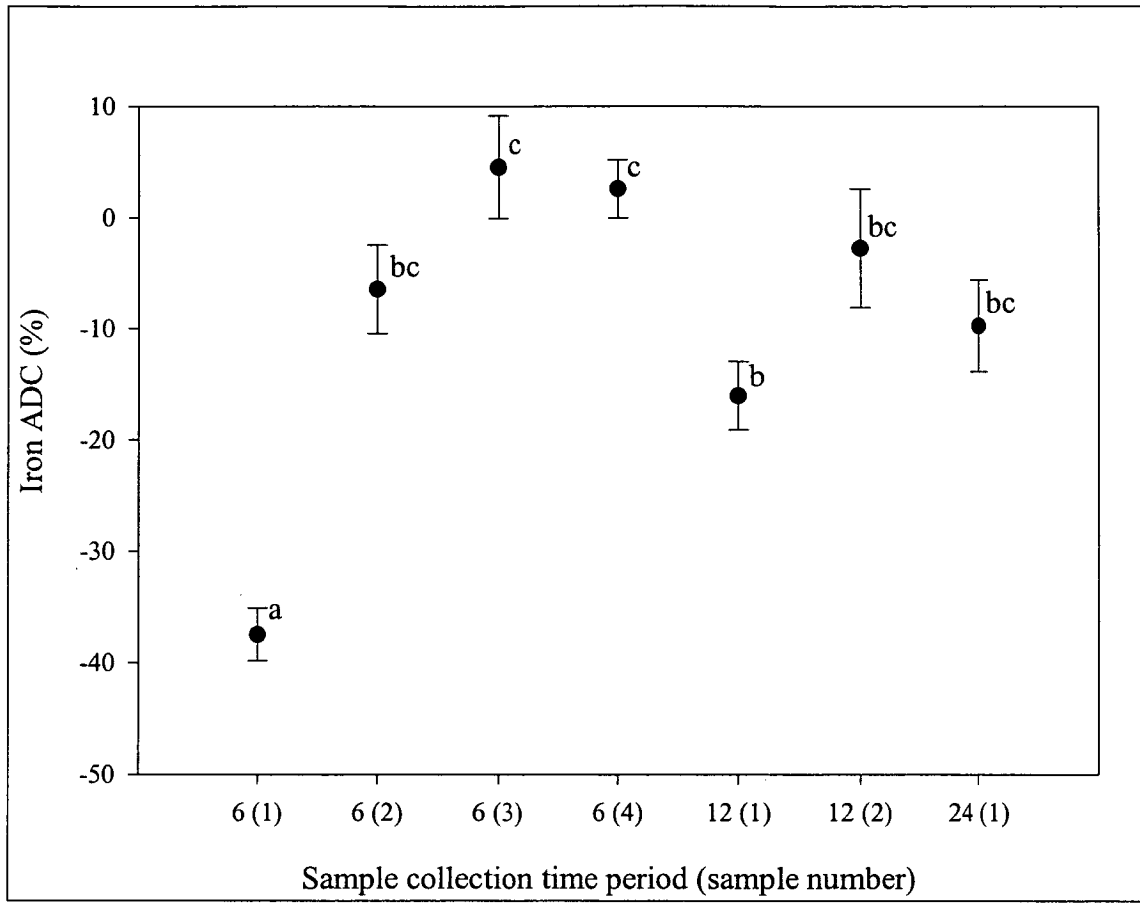

Figure 2.6 Mean (± SEM, $n = 6$) iron ADC (%) calculated from faecal samples collected at differing lengths of time (6 h, 12 h and 24 h) over a 24-h period of collection. Sample collection period means with the same superscript were not significantly different (Tukey's HSD).

2.4 Discussion

2.4.1 Marker type and concentration and ADC calculation

Yttrium oxide can be used to effectively determine ADC for minerals and trace elements in Atlantic salmon feed. It produces recovery rates in excess of 96% from feed samples, is slightly soluble in weak acids but not absorbed by salmon and does not interact with the elements analysed (Hillestad et al., 1999; Austreng et al., 2000). The ease of wet decomposition of samples with concentrated nitric acid in an open system in the present study agree with those from Austreng et al. (2000). This method of sample decomposition prevented recovery errors as compared to chromium oxide, which required decomposition with a stronger acid. Yttrium oxide has also gained popularity for determining the digestibility of macronutrients (Sugiura et al., 1998b; Hillestad et al., 1999; Carter et al., 2003). The feed marked with Y_2O_3 at 0.1% and the respective faecal samples, had concentrations of yttrium within the working range of the element when analysed with ICP-OES (Vandecasteele & Block, 1993). For these reasons this marker and concentration was chosen for further analyses of the effect of faecal sampling time on ADC in the second experiment, and is recommended for future research with minerals and trace elements.

Chromium oxide was not effective at determining ADC for minerals and trace elements at the relatively low levels of inclusion used in this experiment. It was not possible to completely decompose chromium oxide with nitric acid, making it impossible to accurately determine marker concentrations with ICP-OES. This is the reason for using the optical density method of determining chromium content in feed and faecal samples (Furukawa & Tsukahara, 1966). This method achieved reasonable

recovery rates of 106.8 and 96.8% for inclusion levels of 1.0% and 0.1%, but produced results of 140% and 570% for the lower inclusion rates, and this coincides with the 1 – 6% inclusion level suggested by Furukawa and Tsukahara (1966) for determining ADC in fish using this method. ADC generated by the optical density method may generate additional variance as the chromium concentrations are only an approximation of the chromium content with regard to the minerals as determined by ICP-OES, resulting from different decomposition processes that give rise to decomposition matrix effects (Scott, 1978). In addition, the method also employs the use of hazardous perchloric acid, which requires considerable safety precautions.

There are numerous reasons for not using chromium in mineral and trace element digestibility studies. Fernandez et al. (1999) demonstrated that chromium oxide altered the utilization of mineral salts in gilthead sea bream, and it is known to have biological and metabolic effects in Arctic charr (Ringo, 1993), channel catfish (Ng & Wilson, 1997), and tilapia (Shiau & Liang, 1995; Shiau & Shy, 1998). It was unclear what portion of the differences in ADC calculated were due to chromium content, if any. Shiau and Liang (1995) report that the concentration of chromium oxide affected ADC calculations for ingredients and growth in tilapia grown in static ponds, but Ng and Wilson (1997) proposed this was a result of chromium leaching from feed and faeces and could have been accumulated by the fish from the water. Tacon and Rodrigues (1984) suggested that chromium included at 2% has a different rate of passage through the digestive tract relative to digesta, but report no ratios of marker to digesta to verify this. In the present study, it is obvious that marker concentration affected ADC calculation, possibly resulting from differences in passage through the gastrointestinal tract relative to other elements. There were differences in the ratio of

chromium to minerals in the faeces. The rate of recovery of the chromium from feed and faecal samples has an impact on the ADC calculated from the two levels of chromium measured. There are no means to validate the recovery of chromium from the faecal material, which presents a different acid digestion matrix as compared to feed samples, being composed of greater concentrations of material that the fish could not digest. Chromium has displayed poor recovery rates when using wet digestion methods with various acids. Riche et al. (1995) recovered only 13-14% when wet-ashing samples with concentrated nitric acid under pressure with a microwave oven. Using a mixture of hydrochloric-nitric acid, Austreng et al. (2000) recovered 53%. Leid et al. (1982) report recovery of only 40% when using a mixture of nitric-perchloric acid in the sample decompositions, although they achieved recoveries of 98% using concentrated sulphuric acid when analysing chromium from samples decomposed by the micro-Kjeldahl technique. Sulphuric acid is not an acceptable acid when decomposing samples for mineral and trace element analyses, due to sulphur contamination and interference with the measurement of other elements within a sample (Vandecasteele & Block, 1993). Scott (1978) identified prolonged heating and adsorption on to silica residue as causing decreases in the amount of metal extracted when decomposing sediment samples with a mixture of nitric-perchloric acid. Researchers suggest chromium oxide is an acceptable marker for macronutrients (Austreng, 1978; Ng & Wilson, 1997), with greater repeatability than other external markers (Weatherup & McCracken, 1998). However, given the number of problems with the marker and possible effects on digestion of carbohydrates and utilisation of minerals in other species chromium oxide is not recommended for calculating ADC for minerals and trace elements.

The ADC derived from the internal marker AIA differ significantly from those obtained using Y_2O_3 and Cr_2O_3. Morales et al (1999) found that AIA (0.5% diatomaceous silica) produced ADC for protein significantly higher than Cr_2O_3, but Tacon and Rodrigues (1984) report significantly lower ADC from AIA (0.5% acid-washed sand) when compared to Cr_2O_3. In the present experiment AIA was not added to the feed, but used as an internal marker. The ratio of marker in the feed to marker in the faeces was 0.367 for AIA, 0.208 for Y_2O_3 0.1% and 0.205 for Cr_2O_3 1.0%. The differences could have resulted from digestion of AIA, differences in rate of passage or a failure to collect AIA in faecal samples. Additionally, the samples used to determine AIA concentrations cannot be used for ICP-OES analyses to generate mineral and trace element concentrations for ADC calculations, and this information must be obtained from a different sample.

There was some difficulty in determining the ADC for various trace elements with concentrations found in the samples too low to provide accurate information (aluminium, arsenic, boron, cobalt, molybdenum, selenium and tin). For these ultra-trace elements it will be necessary to find alternative methods of analysis, to determine ADC accurately. It is possible to use alternative methods of analysis, such as fluorescence spectrophotometry for selenium (Koh & Benson, 1983), or examine the bioactivity of proteins reliant on specific elements to gauge their bioavailability (Shellow et al., 1985).

2.4.2 Effect of sampling time period on ADC calculation

The concentration of yttrium found in the faeces collected every 6 h varied significantly over a 24 h period. There was a rise and fall of yttrium observed over 24 h, with the greatest concentrations observed between 6 h and 12 h and the lowest concentration observed in the final 6 h (Fig. 2.2). Some ADC values determined from the different sampling periods were significantly different, and those from the last 6 h sample often differed from the samples. An examination of the ratios of Y_2O_3 to mineral content of the faeces for each 6 h sample reveals that there are differential rates of passage with the marker through the gastrointestinal tract for iron, magnesium and manganese. These were the only elements to show definite trends in these ratios, although there were significant differences for sampling periods for many elements. For those elements that show significant differences in ADC, but no difference in the ratio of marker to element in the faeces, other factors must be involved, such as analytical variance or other biological sources of error not associated with passage of an element through the gastrointestinal tract.

Those elements that show the greatest significant differences between the ADC derived from the 6 h samples, over the 24 h sampling period, were iron, magnesium and manganese. There may be several factors involved in the variation in ADC determined, such as particle size and synergistic and antagonistic interactions with other nutrients within the feed (Andersen et al., 1997; Storebakken et al., 2000; Apines et al., 2001). The information provided by these observations show that faeces must be collected over at least 18 h for determining the true ADC of these minerals. This makes it difficult to identify the required concentration of each

element necessary to prevent nutritional deficiencies, as determined from these sampling methods. The need for a representative sample (Maynard & Loosli, 1969) has been identified, and these results indicate that a 24 h samples should fulfil this role, providing observations accurate enough to make supplementation of minerals and trace element and feed formulation more efficient.

Future experiments should consider the relatively small differences in treatments on the statistical power of experiments determining trace element ADC (Searcy-Bernal, 1994). The statistical power of the experiment varied with each element, as mean concentrations and ICP-OES detection ranges varied. Differences in ADC determined from a small number of replicates make it difficult to categorically state that one method of analysis is better than another with regard to "ultra trace" elements, such as molybdenum and cobalt. However, large differences in treatments could results in significant interactions with other ingredients or elements in the feed, and care should be taken to ensure that reference feeds and test ingredients or treatments contain equal concentrations of the element under consideration (Forster, 1999).

There are concerns about ADC derived from various experiments using different digestibility markers and methods of collecting faeces. There is a need to standardise the methods employed in digestibility studies (Storebakken et al., 1998), particularly with minerals and trace elements. Hillestad et al. (1999) agree stating, "no standard method for determination, verification or invalidation of the digestibility of the nutrients in commercial feed is available." It is clear that Cr_2O_3 will be used less frequently for calculating ADC in aquafeeds, and Austreng et al. (2000) have demonstrated that oxides of yttrium, ytterbium and lanthanum are acceptable

substitutes. Analysing samples with ICP-OES has the benefit of providing mineral and trace element concentrations relative to the marker within one sample. Wet digestion of samples in nitric acid at relatively low temperature (100 °C) after freeze-drying limits the loss of elements through volatilization or deposition on vessel surfaces (Vandecasteele & Block, 1993). One drawback to the use of ICP-OES analysis is the cost, which is somewhat greater relative to other methods. However, the need for multiple analyses by alternative methods, such as flame atomic absorption, to generate the number of measurements provided by one ICP-OES analysis offsets any potential savings. The method of faecal collection is the most problematic, as there are a number of practical considerations when conducting digestibility experiments. Collecting faeces using a modification of the Guelph system provided sufficient sample for analysis at this facility, but this may not be the case elsewhere. Collections using settlement should be conducted over at least 18 h, to provide a representative sample which accounts for differences in rates of passage for minerals such as iron, magnesium and manganese.

2.5 Conclusions

Yttrium oxide had many advantages over chromium oxide and AIA for determining mineral and trace element ADC. Yttrium oxide, included as low as 0.1%, was easily analysed with ICP-OES, and provides marker, mineral and trace element concentrations for an individual sample. However, yttrium oxide and external markers in general may not be accurate for measuring "ultra-trace" elements, such as cobalt and molybdenum, and the information gained via ICP-OES may not

represent the true bioavailability of some elements. Further research will be necessary to identify methods other than the use of digestibility markers and faecal collection to determine both the ADC and the bioavailability of these trace elements.

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Chapter 3

Assessment of three faecal collection methods for the determination of mineral and trace element apparent digestibility coefficients in Atlantic salmon (*Salmo salar*, L.) in seawater

Abstract

The effect of collecting faecal samples via settlement, stripping and dissection on the determination of the apparent digestibility coefficients (ADC) of mineral and trace elements in Atlantic salmon held in seawater was assessed. Feed and faecal samples were analysed for the concentration of minerals and trace elements by inductively coupled plasma optical emission spectroscopy and ADC calculated using acid insoluble ash as an internal digestibility marker. Collection of faeces by settlement, stripping and dissection produced 507.8, 214.0 and 209.8 mg of faecal dry matter (DM) per kg of fish sampled respectively. Settlement collection produced significantly higher ADC for calcium, magnesium, phosphorus, copper and manganese. Dissection and stripping resulted in significantly higher ADC for iron, potassium and boron. Faecal samples collected by dissection and stripping produced mean sodium ADC significantly higher than those collected by settlement. Stripping or dissection, while yielding lesser amounts of faecal material, would be the preferred means of collecting faeces for mineral and trace element ADC determinations in Atlantic salmon held in seawater. It was apparent that some nutrients were leached out of the faecal samples and others, present in large quantities in seawater, contaminated samples. Stripping and dissection prevented contact of the faecal samples with urine or seawater, limiting that contamination.

Keywords: Apparent digestibility coefficients Atlantic salmon; faecal collection method; minerals and trace elements

3.1 Introduction

Atlantic salmon (*Salmo salar* L.) and other aquaculture finfish species spend a large proportion of their production cycle in seawater, and it is important to understand the impact this environment may have on the assessment of mineral and trace element apparent digestibility coefficients (ADC) values. Atlantic salmon can ingest sufficient quantities of calcium, magnesium, sodium and potassium from seawater (Lall, 1989), but copper (Lorentzen et al., 1998; Berntssen et al., 1999), iron (Waagbø et al., 1996; Andersen et al., 1997; Maage & Sveier, 1998), manganese (Maage et al., 2000), phosphorus (Åsgård & Shearer, 1997; Baeverfjord et al., 1998), selenium (Poston & Combs, 1979; Bell & Cowey, 1989; Maage et al., 1989; Lorentzen et al., 1994), and zinc (Maage & Julshamn, 1993; Maage et al., 2001) must be provided in feed to prevent deficiencies in these essential elements. Therefore, salmonid feeds are often supplemented with mineral pre-mixes, usually in excess of requirements (Lall, 1989; Watanabe et al., 1997). It would be advisable to end this practice of over-supplementation because as Atlantic salmon production reaches commodity status and the cost of feed per unit of production and the cost of over-supplementation will have to be taken into account (Hardy, 2000). To advance research into mineral and trace element nutrition in Atlantic salmon reared in seawater, it is necessary to find a quick, simple and reliable method of faecal collection.

Research has been conducted investigating the effects that different methods of faecal collection have on the digestibility of macronutrients in salmonids (de la Noüe & Choubert, 1986). The effect of faeces collection method on nutrient leaching have been identified (Windell et al., 1978; Smith, 1980; Satoh et al., 1992), and new

mechanisms for faecal collection (Choubert et al., 1982; Vens-Cappell, 1985) have been developed in attempts to limit the effect of leaching. However, it is not practical to use these methods to collect faeces from Atlantic salmon held in sea cages via settlement. Consequently, faecal samples are often collected by abdominal massage, commonly referred to as "stripping", or dissection (Storebakken et al., 1998a; Percival et al., 2001; Vandenberg & de la Noüe, 2001). As such, it is important to examine the effect these methods of faecal collection and the role seawater composition may have on mineral and trace element ADC determinations. In addition, most commercial feeds do not include external markers for determining ADC. Therefore, internal markers must be used to determine ADC. Acid insoluble ash (AIA) is a common internal marker, and offers a useful alternative to the metal oxides often used (Tacon & Rodrigues, 1984; Morales et al., 1999). The aim of this experiment was to determine the effectiveness of three methods of faecal collection for providing a sufficient amount of faecal DM for analysis to produce mineral and trace element ADC in Atlantic salmon held in sea water. This study also investigated the effect of faecal collection on the mineral and trace element content of the faecal samples obtained.

Unfortunately, sufficient faecal samples were not collected during the present experiment using the stripping and dissection method to enable the determination of AIA from each of these methods. Therefore, the AIA content of faeces collected via settlement were used to calculate ADC for these methods of faecal collection. The consequences of this are that the ADC for minerals and trace elements for these methods of collection have been determined from those values obtained form settlement collection. This assumes there would be no significant differences in the

AIA content of these faecal samples as compared to samples obtained via settlement collection.

3.2 Materials and methods

3.2.1 Fish and experimental approach

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Eighty Atlantic salmon (*Salmo salar*, L.) were allocated to 15 of the 24 300-L tanks comprising the experimental system, held in a constant environment room maintained at 18 h light: 6 h dark photoperiod and 15.0 ± 0.05 °C water temperature. Filtered (0.2 micron) seawater (salinity: 33 ppm) was supplied to the system, which included three settlement tanks, a biofilter and a foam fractionator to remove nitrogenous waste and solids from the recirculated seawater, with a continuous replacement of approximately 10% per day. Water parameters (salinity, dissolved oxygen, oxygen saturation, chlorine, pH, ammonia, nitrate and nitrite) were monitored to ensure water quality remained within limits recommended for Atlantic salmon (Wedemeyer, 1996). The system supplied water to each tank at an average flow rate of 6 l min^{-1}. The fish were acclimated to the experimental feed over a one-week period, and fed to satiation twice a day for a further three weeks. The extruded feed (4.0 mm) was produced under commercial conditions by Skretting Australia (Cambridge, Tasmania, Australia), and stored in a cold room at 2.7 °C until required.

Prior to the start of the experiment, fish were anaesthetised (50 ml l^{-1}, benzocaine) and weighed to the nearest 0.1 g. Seventy-five of the fish were then redistributed to 15 of the tanks, 5 to each tank, ensuring that there were no significant differences in mean body weight for each tank (404.1 ±70.3 g). The biomass of each group of five fish ranged from 1753 to 2298 ±169 g.

3.2.2 Faecal collection

Faeces were collected using a modification of the Guelph design (Cho & Slinger, 1979), as described in Carter and Hauler (2000) (see Chapter 2, Fig. 2.1), by manual stripping and dissection as described by Percival et al. (2001). Each type of collection method was allocated randomly between the tanks in blocks of three down the three rows of the experimental system. Stripping was conducted as described by Percival et al. (2001), with the following modification: faeces were expressed onto a white cutting board held at such an angle as to allow urine to drain away from the faecal material. Collection by stripping and dissection occurred hourly, starting at 10 h after feeding. Each block of tanks was chosen at random until all five blocks had been sampled. All settlement faeces were collected starting 2 hours after feeding, after all faecal collectors were cleaned of any uneaten feed. Faecal samples were also collected from all tanks for 18 h via settlement on the day prior to the randomised sampling to provide sufficient faecal material for acid insoluble ash determinations. All faecal samples were frozen immediately after collection and stored at -4 °C. When all the faecal samples collected had frozen completely, the entire set were

freeze-dried at -10 to -12 °C and -100 to -110 kPa pressure until all samples had reached a constant weight.

3.2.3 Analytical procedures

Standard methods were used to determine dry matter (oven dried at 100 °C to a constant weight), gross energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid) and nitrogen (Kjeldahl using selenium catalyst). Feed and the faecal samples collected by settlement were analysed for acid insoluble ash according to Atkinson et al. (1984). There was insufficient faecal material collected from stripping and dissection to calculate the AIA content of these samples. Therefore, a decision was made to use the mean AIA content from the faeces collected via settlement to derive the ADC for the other methods.

3.2.3.1 Sample decomposition and elemental content analyses

Approximately one gram of the freeze-dried feed or faeces were subjected to wet-decomposition at 100 °C with 5 ml of high purity, concentrated nitric acid (Aristar Grade, 16 M HNO_3). If required, an additional 5 ml of nitric acid was used in the decomposition of faecal samples, to prevent charring. After decomposition the samples were made up to a volume of 50 ml with purified, de-ionised water. Samples were diluted a further 10-fold to improve the determination of highly concentrated minerals, such as phosphorus and calcium.

All samples were analysed for a range of minerals and trace elements using ICP-optical emission spectrophotometry (ICP-OES; Thermo Jarrell-Ash IRIS Axial ICP-OES) at the Central Science Laboratory (University of Tasmania, Hobart, Tasmania, Australia). The mean mineral and trace element content of several blank samples, containing only the concentrated nitric acid, were subtracted from every element in each sample. These blank samples were included in each run to assess the matrix effects of decomposition on ICP-OES analysis. Samples of dogfish muscle tissue (DORM-2, National Research Council, Canada) were used as quality controls for the analytical procedures, including the acid decomposition process. The mean DORM-2 trace element concentrations were within 5% of certified values for all samples analysed.

Those elements commonly referred to as trace elements are found in concentrations of less than 100 mg kg^{-1} of biological samples (Vandecasteele & Block, 1993), and in this experiment were aluminium, arsenic, boron, barium, cadmium, cobalt, copper, manganese, molybdenum, nickel, lead, selenium, silicon, tin, vanadium, yttrium and zinc. The remainder, calcium, iron, potassium, magnesium, sodium, phosphorus, and sulphur, were referred to as minerals.

3.2.3.2 Apparent digestibility coefficients

Mineral and trace element ADC were determined for samples from each collection method. However, the limited amount of faecal material collected from the stripping and the dissection methods meant that mineral analysis could only be conducted on

pooled samples taken from all the tanks using that particular faecal collection method. ADC were calculated, for each mineral and trace element that was present in reportable concentrations, according to the following equation:

$$ADC(\%) = 100 - \left[\frac{[M]feed \times [N]faeces}{[M]faeces \times [N]feed}\right] \times 100 \qquad [3.1]$$

(Maynard & Loosli, 1969) where [*M*] was the concentration (mg kg^{-1}), of the digestibility marker (AIA), and [*N*] the concentration of the nutrient (mg kg^{-1}).

3.2.4 Statistical analyses

The statistical methods described in Underwood (1981) and Zar (1984) were applied using SPSS v. 10.0 software (SPSS, 2000). All ADC data were tested for normality (Shapiro-Wilk), and, where applicable, ADC data was arcsine transformed with the following equation (Zar, 1984):

$$ADC' = \arcsin\sqrt{ADC} \qquad [3.2]$$

Some calculated ADC data were negative and it was not possible to arcsine transform these values. The data, including arcsine transformed ADC, were analysed using a one-way analysis of variance (ANOVA) to compare the difference between means of the mineral and trace element ADC and faecal mineral content resulting from each method of faecal collection. Tukey's honestly significant difference test (Tukey's

HSD) was used for multiple comparison of means for all data. Significance for all statistical tests was accepted at probability levels of 0.05 or less.

3.3 Results and discussion

3.3.1 Faecal collection methods

The feed contained sufficient amounts of crude protein (nitrogen) and energy, similar to other commercial feeds used in the Tasmanian salmon industry, and the concentrations of mineral and trace elements in the feed (Table 3.1) were in excess of those required in salmonids for calcium, copper, iron, potassium, manganese, phosphorus, sulphur, selenium and zinc (Lall, 1989; NCR, 1993).

The three methods of faecal collection differed significantly in the amount of faecal material provided, and while the settlement and stripping methods of collection provided similar results over time, the amount of faecal material obtained by dissection appears to decrease over time (Figure 3.1). Approximately one gram of dry faecal material is required to provide sufficient material for ICP-OES analysis of trace elements. Settlement collection yielded 507.8 mg faecal DM per kilogram of fish sampled (5217 mg DM total), and provided the required amounts of faeces for ICP-OES and AIA analysis. The stripping method provided 243 mg faecal DM per kg of fish sampled (2502 mg DM total), which was comparable to the 262 mg faecal DM per kg of fish sampled obtained by Vens-Cappell (1985) using rainbow trout. Some attempts to strip fish yielded no faeces at all. Dissection yielded 209.8 mg faecal DM per kilogram of fish sampled (2046 mg DM total).

Table 3.1 Chemical composition (±SEM, $n = 4$) of the commercial Atlantic salmon feed

Chemical composition	Mean (±SEM)
Dry matter (g kg^{-1})	911.0 (0.6)
Gross energy (MJ kg DM^{-}	22.7 (0.2)
Crude protein (g kg^{-1} DM^{-}	408.7 (0.9)
AIA (mg kg^{-1} DM)	2083 (214)
Minerals	**g kg^{-1} DM**
Ca	18.72 (0.28)
Fe	0.19 (0.01)
K	7.29 (0.13)
Mg	2.18 (0.01)
Na	7.28 (0.16)
P	13.02 (0.21)
S	34.24 (1.68)
Trace elements	**mg kg^{-1} DM**
Al	46.80 (2.7)
As	3.76 (0.4)
B	13.06 (0.8)
Cd	1.04 (0.1)
Co	2.31 (0.1)
Cr	0.94 (0.2)
Cu	17.81 (0.8)
Mn	79.95 (2.0)
Mo	1.15 (0.2)
Ni	1.39 (0.1)
Pb	2.54 (0.1)
Se	1.33 (0.6)
Si	5.32 (1.2)
Y	0.63 (0.7)
Zn	140.59 (1.3)

The experimental feed was sourced from Skretting (Cambridge, Tasmania, Australia).

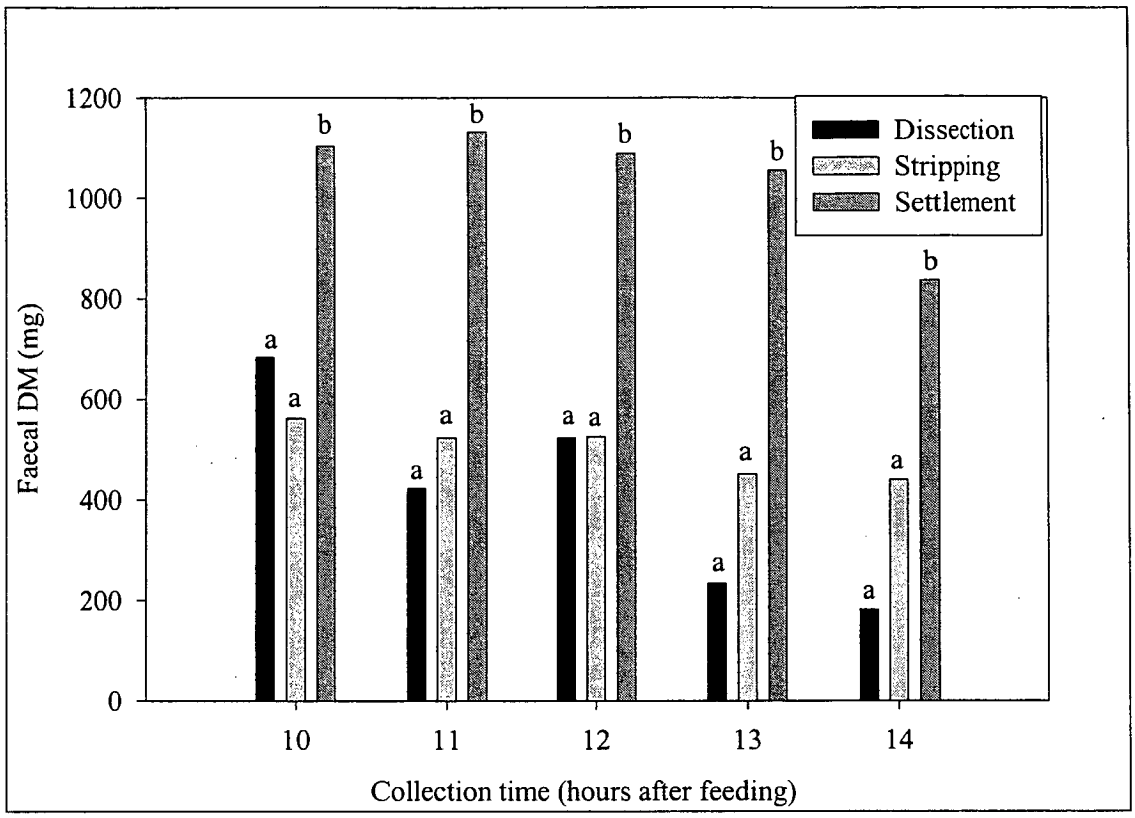

Figure 3.1 Faecal DM (mg) collected from Atlantic salmon over time from each method of faecal collection. The mean faecal DM (mg) differed significantly (F = 29.74, df = 2, P <0.001), and those faecal collection methods with different superscripts were significantly different (Tukey's HSD).

Unfortunately, dissection and stripping failed to provide sufficient sample (0.75-1.00 g) for individual nutrient analysis for each tank, therefore, the samples were pooled. The pooled samples provided enough material for three ICP-OES analyses for each sampling method. The AIA content of feed and faeces collected via the additional settlement collection were 2083 ± 214 mg kg^{-1} (n = 4), and 7196 ± 452 mg kg^{-1} (n = 14) respectively. To provide sufficient quantities of faecal material for at least one ICP analysis per tank (750 mg), would have required almost twice as much fish biomass, an additional 2.7 and 1.3 kg of fish for the dissection and stripping tanks. To provide sufficient faecal material for one AIA analysis per tank would have required an additional 15 and 19 kg of fish for stripping and dissection respectively. Alternatively, we could have stripped the fish several times to obtain sufficient faecal material (Percival et al., 2001).

There are several concerns regarding the use of stripping and dissection as methods of faecal collection. The effectiveness of stripping depends a great deal on several factors including: stripping technique (Percival et al., 2001), the amount of pressure applied, the level of anaesthesia and timing of collection with regard to last feeding (Vandenberg & de la Noüe, 2001). A high proportion of variability in total faecal sample collected by stripping must be attributed to the personnel applying the technique. Initial attempts to follow the method described by Percival et al. (2001) indicated that a modification was required to effectively collect samples. It would be more difficult to standardise this method than others which rely less on human interaction with the fish such as settlement, dissection and mechanical sieving. Stripping is often done using fish that are larger than those in this experiment or,

when using smaller fish, samples are taken from several sessions and pooled to provide sufficient material for the calculation of the ADC (Table 3.2). Multiple sampling sessions are often spaced several days apart to limit any possible impact of handling on fish and feeding. Dissection provides no opportunity for multiple sample of the same group of fish, but is less susceptible to contamination. Percival et al. (2001) found that while there were some difficulties associated with stripping fish, such as contamination with urine and/or mucus, it was a suitable method for calculating ADC for energy and protein. In the present study urine contamination was limited by expressing the faeces onto a board held at an angle allowing the majority of the urine to drain away from the faecal sample. It was assumed that cleaning the fish with a paper towel prior to expressing faeces would limit mucus contamination, and it is unlikely this limited contamination would impact mineral and trace element ADC calculations. The ADC for copper and iron calculated from the dissected and stripped samples may differ from the settlement samples due to incomplete digestion and absorption of minerals. This "early" removal of faecal material is commonly cited as a reason for differences in ADC calculated for other dietary components (Windell et al., 1978; Percival et al., 2001).

There were advantages and disadvantages to the use of settlement for the collection of faecal material in seawater for the calculation of mineral and trace element ADC. Settlement collection provided sufficient amounts of faecal material for analysis. Settlement collection permitted multiple sampling without the need to repeatedly handle the fish. However, it is not practical to collect faeces from Atlantic salmon in sea cages via settlement. Several researchers have (Windell et al., 1978; Smith, 1980; Cho et al., 1982; Vens-Cappell, 1985) cited the loss of soluble material from Table

3.2 Fish sizes, total biomass and number of stripping sessions required by salmonid digestibility experiments

Salmonid species[1]	Fish weight range (g)	Fish biomass (g) per tank	Number of samplings	Source
AS	112	2240	1	(Baeverfjord et al., 1998)
AS	200	10,000	1	(Refstie et al., 1998)
AS	4600	32,200	1	(Thodesen et al., 2001a)
AS	200	2400	1	(Storebakken et al., 1998a)
AS	600	18,000	1	(Refstie et al., 1999)
AS	100	1590	1	(Storebakken et al., 1998c)
AS	940	18,800	2	(Storebakken et al., 2000)
AS	540	16,200	2	(Hillestad et al., 1999)
AS	1000	20,000	2	(Hillestad et al., 1999)
AS	15	1,350	4-5	(Vielma & Lall, 1998)
RT	275	1650	1	(Sugiura et al., 1999)
RT	153	1989	1	(Sugiura et al., 2001)
RT	700	18-20 fish	2	(Storebakken et al., 1998b)
RT	165-180	2,310-2,520	3	(Tacon & Rodrigues, 1984)
RT	136-205	12-37 fish	every 3-5 days*	(Aksnes et al., 1996)
RT	100-400	12-35 fish	every 3-5 days*	(Aksnes & Opstvedt, 1998)

[1] Species of salmonid: AS = Atlantic salmon, RT = rainbow trout.

* Fish were sampled every 3-5 days until sufficient faecal material was collected.

leaching as the main criticism for the settlement collection method. Stripping often results in lower ADC for protein than those obtained from settlement collection (Vens-Cappell, 1985; Hajen et al., 1993a). Our results indicate that it may be a source of error in calculating ADC for calcium, potassium, magnesium, sodium, phosphorus and copper. Samples collected by settlement reacted quite vigorously to the acid decomposition process, and required repeated mixing with a vortex mixer to prevent the loss of samples. This was most likely related to the high proportion of available minerals (sodium, magnesium and potassium) present in the sea water residue remaining after freeze drying the sample. Water was collected along with the faecal samples collected via settlement, to ensure material leached out of the faeces in the water immediately surrounding the sample, evidenced by the presence of discoloured water in the collection jar, was retained. The extremely negative ADC for sodium calculated from those faeces collected by the settlement method indicate the influence of seawater contamination and highlights the benefit of collecting faecal samples with methods that eliminate or limit contact with sea water (Choubert et al., 1982; Vens-Cappell, 1985).

3.3.2 ADC

There were significant differences in the ADC determined for all minerals except sulphur (Table 3.3). Potassium and sodium were present in significantly higher concentrations in the faecal samples collected via settlement. The only trace elements to show any significant differences between the collection methods were boron, copper and manganese (Table 3.4). The ADC calculated from stripping and

Table 3.3 Effect of faecal collection method on mean (±SEM) faecal mineral concentrations (mg kg^{-1})

Element	Collection method			F value ($df = 2$)	P
	Dissection ($n = 3$)	Stripping ($n = 3$)	Settlement ($n = 5$)		
Ca	76324.43^{b} (7639.33)	73774.55^{b} (6953.20)	57356.20^{a} (5571.80)	10.23	0.006
Fe	609.11^{ab} (13.98)	657.38^{b} (22.65)	566.24^{a} (50.97)	5.33	0.34
K	1705.05^{a} (12.90)	1220.20^{a} (48.65)	5509.80^{b} (580.52)	133.10	<0.001
Mg	40147.24^{b} (4177.46)	36121.08^{b} (4330.37)	23882.27^{a} (3256.34)	20.14	0.001
Na	18895.05^{a} (791.84)	18127.43^{a} (772.60)	123926.12^{b} (12779.44)	184.88	<0.001
P	29492.36^{b} (1106.37)	25862.50^{ab} (1255.67)	20658.17^{a} (3674.79)	10.34	0.006
S	34156.08 (15746.08)	32572.63 (14778.70)	41159.24 (1416.49)	0.72	ns

Means with the same superscript are not significantly different (Tukey's HSD).

Table 3.4 Effect of faecal collection method on mean (±SEM) faecal trace element concentrations (mg kg^{-1})

Element	Collection method			F value ($df = 2$)	P
	Dissection ($n = 3$)	Stripping ($n = 3$)	Settlement ($n = 5$)		
Al	116.76 (19.27)	99.45 (9.70)	77.81 (33.33)	2.19	ns
B	36.43[a] (4.34)	35.91[a] (6.69)	88.64[b] (7.08)	91.63	<0.001
Cd	8.32 (11.52)	1.28 (0.72)	14.93 (6.12)	3.40	ns
Co	5.52 (1.91)	6.52 (2.55)	5.33 (3.61)	0.15	ns
Cu	29.47[b] (1.08)	30.38[b] (2.65)	18.45[a] (2.96)	28.07	<0.001
Mn	186.63[b] (5.11)	186.13[b] (14.41)	135.70[a] (25.33)	9.23	0.008
Si	39.30 (17.53)	44.63 (15.94)	53.22 (3.42)	1.33	ns
Y	5.03 (3.14)	4.58 (4.52)	4.94 (10.19)	0.57	ns
Zn	185.23 (17.91)	179.72 (20.97)	148.06 (14.67)	5.50	ns

Means with the same superscript are not significantly different (Tukey's HSD).

dissection were similar to those reported by Storebakken et al. (2000) and Thodesen et al. (2001c) for potassium, calcium, copper, iron, magnesium, manganese, phosphorus and zinc obtained from stripping fish held in seawater and calculated using the external marker yttrium oxide. The standard error of the mean ADC for cadmium, magnesium, silicon and yttrium, from all three methods of collection were greater than the mean, due to variance in faecal concentrations (Tables 3.5 and 3.6).

The concentrations of arsenic, chromium, molybdenum, nickel, lead, selenium and tin in the faecal samples were below the detection levels of ICP-OES analysis. There were conflicting results provided from two wavelengths used to analyse vanadium. One wavelength indicated concentrations below detection levels and the other indicated concentrations higher than usually seen in faecal samples (per comm., Ashley Townsend 19 Feb 2003). Therefore, ADC were not calculated from either wavelength for vanadium.

The ADC calculated in this study could have been affected by the minerals present in the seawater in two ways. Firstly, the ingestion of seawater could have affected the ADC calculated for all methods. The seawater in the system could provide significant amounts of sodium (1077 mg d^{-1}), magnesium (129 mg d^{-1}), sulphur (90.5 mg d^{-1}), calcium (41.2 mg d^{-1}) potassium (38 mg d^{-1}) and boron (0.44 mg d^{-1}) when compared to the amounts provided by feed (Bearman, 1989; Thodesen et al., 2001b). However, it was not possible to measure the amount of seawater ingested by the fish in this study. Secondly, mineral contamination of faecal samples could have affected the mineral content of the faeces collected via settlement. The samples obtained in this study were not subjected to centrifugation (Hajen et al., 1993a; Hajen et al., 1993b)

Table 3.5 Effect of faecal collection method on mean (±SEM) mineral ADC (%)

Element	Collection method			F value ($df = 2$)	P
	Dissection ($n = 3$)	Stripping ($n = 3$)	Settlement ($n = 5$)		
Ca	-65.9[a] (46.0)	-60.4[a] (38.1)	-24.7[b] (14.7)	10.21	0.006
Fe	-27.7[ab] (1.4)	37.8[a] (3.8)	-18.7[b] (11.4)	5.33	0.034
K[1]	90.5[b] (0.0)	93.2[b] (0.0)	69.2[a] (1.1)	211.07	<0.001
Mg	-649.2[a] (1012.8)	-574.0[a] (1088.3)	-345.7[b] (369.2)	20.14	0.001
Na	-5.5[b] (3.3)	-1.2[b] (3.1)	-592.2[a] (509.5)	184.85	<0.001
P	7.9[a] (2.0)	19.2[ab] (2.6)	35.5[b] (13.2)	10.32	0.006
S	59.4 (58.4)	61.3 (51.4)	51.1 (0.3)	0.722	ns

Means with the same superscript are not significantly different (Tukey's HSD).

[1]Data for this element was arcsine transformed prior to statistical analysis.

Table 3.6 Effect of faecal collection method on mean (±SEM) trace element ADC (%)

Element	Collection method			F-value ($df = 2$)	P
	Dissection ($n = 3$)	Stripping ($n = 3$)	Settlement ($n = 5$)		
Al	-1.6 (46.8)	13.5 (11.9)	32.3 (84.0)	2.19	ns
B	-13.6[b] (30.5)	-11.9[b] (72.5)	-176.3[a] (48.8)	91.73	<0.001
Cd	-226.1 (33983.2)	49.9 (132.0)	-485.3 (5759.3)	3.40	ns
Co	2.6 (188.4)	-15.0 (337.3)	6.0 (405.7)	0.15	ns
Cu	32.6[a] (1.0)	30.6[a] (6.1)	57.8[b] (4.6)	27.94	<0.001
Mn	5.0[a] (1.1)	5.2[a] (9.0)	30.9[b] (16.6)	9.22	0.008
Si	-200.5 (2992.5)	-241.2 (2475.1)	-306.9 (68.2)	1.32	ns
Y	-226.7 (6919.8)	-197.3 (14393.3)	-220.7 (43818.2)	0.01	ns
Zn	46.4 (4.5)	48.0 (6.1)	57.1 (1.8)	5.45	ns

Means with the same superscript are not significantly different (Tukey's HSD).

or "washing" (DeSilva & Perera, 1983) to remove minerals present in the samples as a result of the seawater in the collection vessel, as has been done in other experiments. The potassium, sodium and boron concentrations of the faeces collected with this method were significantly greater than those collected with the other two methods.

Acid insoluble ash was not ideal for calculating the ADC of minerals and trace elements. To accurately determine the concentration of AIA in a singe sample required three grams of dry faecal material, more than the total amount collected from dissection and stripping collection methods. The failure to obtain sufficient faecal material from all the collection methods did not permit us to compare the concentrations of AIA between the methods of faecal collection, and it was assumed the values obtained from the settlement collection would not differ significantly from the other methods. The mineral and trace element ADC were calculated from the mean AIA content of faecal samples that were assessed separately from those used to identify mineral and trace element content. The accuracy of ADC calculations would be improved by using a marker or element which could be analysed at the same time as the minerals and trace elements of a sample with ICP-OES. It is possible that some of the elements present in these samples could be used as internal markers for mineral digestibility, as suggested by Leid et al. (1982), who reported that protein digestibility estimates using gastrointestinal levels of calcium and zinc corresponded well with those made with external markers in Atlantic cod (*Gadus morhau*).

3.4 Conclusions

Relatively large amounts of faecal material are required to use acid insoluble ash as an internal marker and for ICP-OES analysis of elemental concentrations. The settlement method of collecting faecal samples provided the amounts of faecal material required over a short time period, without handling or destroying the fish. However, settlement collection of faeces in seawater allowed minerals and trace elements to either leach out of or be deposited on the sample, resulting in significantly different ADC, depending on the mineral in question. Stripping and dissection are more practical methods of collecting faeces in a commercial production setting and provided equivalent measures ADC for most minerals and trace elements. The problem of obtaining the required amounts of faecal material may be overcome by increasing biomass of fish sampled or conducting multiple sampling sessions.

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Chapter 4

Effect of mineral supplementation on apparent digestibility coefficients and mineral concentrations in tissues of juvenile Atlantic salmon (*Salmo salar* L.) fed fish meal based feeds over 60 days

Abstract

Four experimental feeds were formulated to add minerals and trace elements at 0%, 20%, 40% and 80% of estimated requirements to a reference salmon feed composed primarily of fish meal and wheat flour. Faecal, blood, muscle and combined liver-kidney samples were taken throughout the experiment. Apparent digestibility coefficients (ADC) were calculated from faecal samples taken on days 10, 45 and 60. Varying supplementation had no significant effect on the iron, magnesium, molybdenum and selenium concentrations in the feeds, due to variability in the reference feed ingredients. There were no significant effects on growth parameters of the fish measured after 60 days of feeding. The length of feeding significantly affected ADC calculated for aluminium, molybdenum, phosphorus, lead, sulphur and selenium. Mineral supplementation significantly affected ADC calculated for potassium, magnesium, nickel, phosphorus and selenium. There were significant interactions between mineral supplementation and sampling day in ADC calculated for calcium, magnesium, manganese, molybdenum, phosphorus and silicon. The concentrations of sodium in blood, and copper and selenium in the liver-kidney samples differed significantly by treatment. While significant differences in mineral and trace element concentrations were found in the various tissues sampled, these did not always correlate to the significantly different ADC observed over the same period for those elements. Tissue samples provided much stronger statistical power for identifying small differences between the effects of mineral supplementation.

Keywords: Apparent digestibility coefficients Aquaculture feeds; Atlantic salmon; mineral supplementation; tissue mineral concentrations

4.1 Introduction

Assessment of the effectiveness of an ingredient or supplement to increase the concentration and/or digestibility of minerals and trace element in an aquafeed is often based on the apparent digestibility coefficients (ADC) for nutrients supplied by those ingredients (Hillestad et al., 1999). The current method of determining the ADC of ingredients in finfish aquafeeds is to compare the concentration of nutrients and internal markers in collected faeces to those in the feed (Austreng, 1978; Cho et al., 1982; Hillestad et al., 1999; Austreng et al., 2000). There are limitations with using this method. The foremost, in relation to mineral determination, is contamination of the collected faeces with other matter from the fish or the environment. Secondly, the ADC of an ingredient or supplement does not necessarily indicate bioavailability (Lall, 2002). Thirdly, ADC made at the end of an experiment may not identify changes that occur over time. The ADC of an ingredient are often calculated from faecal samples obtained from faecal collections taken at the end of an experiment (Austreng, 1978; Storebakken et al., 1998; Sugiura et al., 1999), or collected over a period of several days and pooled (Cho et al., 1982; Vens-Cappell, 1985; Hajen et al., 1993a). Sugiura et al. (1998) conducted one of the few experiments that calculated mineral and trace element digestibility throughout an experiment.

Tissue samples can provide a variety of mineral status indicators, representing the long-term and short-term mineral nutritional history of an animal (Lorentzen et al., 1996), and improve the analytical power of an experimental design when included. Whole fish are often used to determine the effect of dietary minerals when estimating

the requirement of an element (Shearer et al., 1994; Åsgård & Shearer, 1997; El-Mowafi et al., 1997; Lorentzen & Maage, 1999; Storebakken et al., 2000; Sugiura et al., 2000a; Green et al., 2002a; 2002b; Apines et al., 2003). However, the effects of mineral and trace element supplementation may not be easily detected in whole fish in the short term, and the analysis of blood, muscle, and liver-kidney samples would aid in distinguishing if trace element deficiencies existed. The analysis of individual tissues, instead of whole fish, will limit the effect of organs that concentrate minerals on elements which could interfere with the accurate detection of trace elements, such as bones and scales which contain significant amounts of calcium and phosphorus.

The aims of this experiment were to calculate sequential ADC for various levels of mineral supplementation in a fish meal based feed, composed of common feed ingredients. The investigation sought to identify the effect of mineral supplementation on tissue mineral and trace element concentrations in a feed where the ingredients provided a substantial proportion of the minerals and trace elements present in the feed. The relationship between mineral supplementation, mineral content and bioavailability was observed in blood, muscle, and liver-kidney samples and related to ADC derived over 60 days.

4.2 Materials and methods

4.2.1. Feeds

Four experimental feeds were formulated from a reference salmon feed (Table 4.1) which included mineral and trace element supplements at four levels: 0%, 20%,

Table 4.1 Formulation and chemical composition of the experimental feeds

	Source[1]	Supplement level			
		0%	20%	40%	80%
Ingredient (g kg^{-1})					
Fish meal (Peruvian)	Skretting	750.00	750.00	750.00	750.00
Wheat flour	Gibson's	104.65	104.65	104.65	104.65
Fish oil (Peruvian)	Skretting	70.00	70.00	70.00	70.00
α-Cellulose	Sigma-Aldrich	62.71	47.04	31.36	0.00
CMC binder	Sigma-Aldrich	10.00	10.00	10.00	10.00
Yttrium oxide	Sigma-Aldrich	1.00	1.00	1.00	1.00
Mineral and vitamin mix	see Table 4.2	1.64	17.31	32.99	64.35
Chemical composition					
Dry Matter (g kg^{-1})		879	887	886	845
Gross energy (MJ kg^{-1} DM)		19.3	19.8	19.7	18.9
Crude protein (g kg^{-1} DM)		476	507	502	496

[1]Ingredients from Skretting and Gibson's were sourced from Cambridge, Tasmania, those from Sigma-Aldrich sourced from Castle Hill, NSW.

40% and 80% of requirements (Table 4.2), based on the mineral content of the fish meal and wheat flour in the reference feed and requirement estimates from various sources (see Chapter 1, Table 1.1 and 1.2). The feed was thoroughly mixed, and included 0.1% yttrium oxide (Y_2O_3), as an external digestibility marker. The feeds were

pelleted at room temperature with a 3.4 mm die (CL-2 laboratory pellet mill, California Pellet Mill Co., San Francisco, U.S.A.). Immediately prior to pelleting, 100 ml of distilled water was added per kilogram of feed. Pelleting proceeded from the feed with the least concentration of mineral supplementation to the greatest, and the mill was cleaned after pelleting each feed. The pelleted feeds were oven-dried at 40°C for over 24 h, allowed to cool, then stored in a cold room at 2.7°C.

4.2.2. Experimental system, fish and sample collection

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Seven hundred Atlantic salmon (*Salmo salar* L.) parr were obtained from Springfield Hatchery (Springfield, Tasmania, Australia), and distributed to 20 tanks in a 24-tank experimental system, at approximately 30 fish per tank. The twenty-four 300-L tanks comprising the experimental system were held in a constant environment room. Water temperature was maintained at a temperature of 15.0 °C. Photoperiod was held constant at 16 h light and 8 h dark. Bio-filtered, freshwater was supplied to each tank in a partial replacement system with a continuous replacement of approximately 10% per day from the municipal water supply. This replacement water entered the system though an activated charcoal

Table 4.2 Mineral and vitamin supplementation of the experimental feeds

Supplement (mg kg^{-1})	Supplement level			
	0%	20%	40%	80%
Potassium phosphate dibasic	0.00	12000.00	24000.00	48000.00
Calcium carbonate	0.00	1400.00	2800.00	5600.00
Sodium chloride	0.00	2000.00	4000.00	8000.00
Magnesium carbonate	0.00	140.00	280.00	560.00
Ferrous sulphate ($FeSO_4$-$7H_2O$)	0.00	40.00	80.00	160.00
Zinc sulphate ($ZnSO_4$-$7H_2O$)	0.00	15.00	30.00	60.00
Manganous sulphate ($MnSO_4$-$4H_2O$)	0.00	16.00	32.00	64.00
Cupric sulphate ($CuSO_4$-$5H_2O$)	0.00	4.71	9.43	18.86
Cobalt sulphate ($CoSO_4$-$7H_2O$)	0.00	1.91	3.81	7.62
Potassium iodide	0.00	0.29	0.58	1.15
Sodium selenate (Na_2SeO_3)	0.00	0.13	0.26	0.53
Vitamin B_{12}	0.00	0.00	0.00	0.01
Stay-C® (L-ascorbyl 2 polyphosphate)	50.00	50.00	50.00	50.00
Calcium D-pantothenate	21.73	21.73	21.73	21.73
Nicotinic acid	10.00	10.00	10.00	10.00
Retinol acetate (500000 IU/g)	4.80	4.80	4.80	4.80
Riboflavin	4.00	4.00	4.00	4.00
Pyridoxine HCl	3.66	3.66	3.66	3.66
Vitamin D_3 powder (400000 IU/g)	6.00	6.00	6.00	6.00
Menadone sodium bisulphate	2.00	2.00	2.00	2.00
Thiamin HCl	1.12	1.12	1.12	1.12
Folate	1.00	1.00	1.00	1.00
d-Biotin	0.15	0.15	0.15	0.15
Choline chloride	1333.33	1333.33	1333.33	1333.33
DL alpha tocopherol acetate	200.00	200.00	200.00	200.00

All mineral and trace element supplements were sourced from Sigma-Aldrich, Castle Hill, NSW, except for Stay-C® which was sourced from Roche Vitamins Australia,

Frenchs Forest, NSW.saturation, chlorine, pH, ammonia, nitrate and nitrite) were filter, to reduce mineral fluctuations. Water parameters (dissolved oxygen, oxygen monitored to ensure water quality remained within limits recommended for Atlantic salmon (Wedemeyer, 1996). The system supplied water to each tank at an average flow rate of 6 l min^{-1}. Water samples of the input and system water were taken weekly and frozen, and at the end of the experiment analysed for mineral and trace element concentrations.

At the start of the experiment the fish were anaesthetised (50 mg l^{-1}, benzocaine), weighed to the nearest 0.1 g and fork length (FL) recorded to the nearest mm. Fish under 20 g in bodyweight (BW) were excluded from the experiment. The fish were redistributed to 20 tanks, at a stocking rate of 30 fish per tank, ensuring that there were no significant differences in mean BW for each tank (27.5 ±0.8 g).

Fish were switched from the reference feed, which had been supplemented at 100% of the mineral and trace element requirements estimated for Atlantic salmon, to the experimental feeds after fasting for 5 days, and were fed 2.0 - 2.5% BW of feed per day split equally between the morning (9:00) and evening (17:00) feeding period. Feeding was set to the lowest tank feeding level monitored on the previous day in order to ensure equal feed intake.

Fish faeces were collected using a modification of the Guelph design (Cho & Slinger, 1979), as described in Carter and Hauler (2000) the modification included the use of a widened portion on the outflow pipe, to facilitate the settlement of faecal material, above a removable, 70-ml, sterile container, put in place for each collection period

(see Chapter 2, Fig. 2.1). Faecal samples were collected on days 10, 45 and 60 from each tank for 18 h, starting one hour after the evening feeding. The faecal collectors were checked to ensure that any uneaten feed was not present prior to the start of collection. Faecal samples were frozen immediately after collection and stored at -4 °C.

Three fish from each tank were removed and blood, white muscle, and liver-kidney samples were taken at days 10 and 60. In addition, blood samples were taken from nine fish on day one. Fish were anesthetised with 10% benzocaine, then bled out. After bleeding, the kidney and liver were dissected and weighed together. Liver and kidney samples were combined to provide sufficient sample mass for analysis. A portion of skin-free, white muscle was taken from approximately 1 cm anterior of the dorsal fin to 1 cm posterior to the dorsal fin, and only muscle dorsal of the midline of the fish was retained. All tissue sampling occurred within an 8 h period, with each treatment block was sampled randomly to avoid any systematic errors, and all samples were immediately frozen at -4 °C, then freeze-dried. The BW and FL of each fish was recorded when sampling tissue on days, 10 and 60, and for the remaining fish on day 60 at the end of the experiment, and used to calculate specific growth rates (SGR) as:

$$SGR\left(\%\, day^{-1}\right) = 100 \times \left[\ln\left(\frac{W_2}{W_1}\right)\right] \times d^{-1} \qquad [4.1]$$

where W_1 and W_2 were the weights (g) at two times and d the number of days.

4.2.3 Analytical procedures

Standard methods were used to determine dry matter (oven dried at 100 °C to a constant weight), gross energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid) and nitrogen (Kjeldahl using selenium catalyst).

4.2.3.1 Sample decomposition and elemental content analyses

All tissue samples from each replicate tank were pooled and processed as a unit. Samples were weighed into acid-rinsed test tubes and decomposed with 5 ml of nitric acid (Aristar Grade, 16 M HNO_3) at 100 °C for 4 hours. Approximately, 1.0 to 1.5 ml of blood was used for analysis. The kidney and liver samples were processed and analysed together.

One gram of each type of sample, including: freeze-dried feed, faeces, muscle and liver-kidney samples, collected from each tank were subjected to wet-decomposition at 100 °C with 5 ml of concentrated nitric acid (Aristar Grade, 16 M HNO_3). If required, an additional 5 ml of nitric acid was used in the decomposition of faecal samples, to prevent charring. Feed and faeces samples were processed at the same time and blood, muscle and liver-kidney were processed together. After decomposition the feed, faeces, muscle, and liver-kidney samples were made up to a volume of 50 ml with purified, de-ionised water, blood samples were made up to 25 ml. Samples were diluted a further 10-fold to improve the determination of highly

concentrated minerals, such as phosphorus and calcium. Water samples were not subjected to acid decomposition and were not diluted prior to analysis.

All samples were analysed for a range of minerals and trace elements using ICP-optical emission spectrophotometry (ICP-OES) at the Central Science Laboratory (University of Tasmania, Hobart, Tasmania, Australia). Blank samples, containing only the decomposition acid, were included to measure the matrix effects of decomposition, which were subtracted from every element in each sample. Mineral and trace element quality control standard samples were used to assess the accuracy of the ICP-OES analysis, and were within 5% of known concentrations for all elements. Samples of dogfish muscle tissue (DORM-2, National Research Council, Canada) were included as reference samples in every decomposition procedure, and the DORM-2 trace element concentrations were within 5% of certified values for all decompositions. Any element found in concentrations below ICP-OES detection limits was not reported.

4.2.3.2 Apparent digestibility coefficients

ADC for each mineral and trace element were calculated, according to the following equation:

$$ADC(\%) = 100 - \left[\frac{[M]feed \times [N]faeces}{[M]faeces \times [N]feed}\right] \times 100 \qquad [4.2]$$

(Maynard & Loosli, 1969) where [*M*] was the concentration (mg kg^{-1}), of the digestibility marker (Y_2O_3), and [*N*] the concentration of the nutrient (mg kg^{-1}).

4.2.4 Statistical analyses

4.2.4.1 Statistical procedures

The statistical methods described in Underwood (1981) and Zar (1984) were applied using SPSS v. 10.0 software (SPSS, 2000). For statistical analysis the experimental unit was the tank; all samples, including pooled tissue samples taken from three fish in one tank were combined for analysis. All ADC data were tested for normality (Shapiro-Wilk), and, where applicable, ADC data was arcsine transformed with the following equation (Zar, 1984):

$$ADC' = \arcsin\sqrt{ADC} \qquad [4.3]$$

Some calculated ADC data were negative and it was not possible to arcsine transform these values. All mineral and trace element concentration data from each type of tissue and ADC data, including arcsine transformed ADC, were analysed using a two way analysis of variance, to analyse the effect of sample day and mineral supplement level. A one-way analysis of variance was used to compare the differences between the mean concentrations of minerals and trace elements present in water samples taken from the system and those taken from the input. Tukey's honestly significant difference test (Tukey's HSD) was used for multiple comparison of means for all data. Pearson's correlation coefficient was used to compare the correlation

coefficients between blood, muscle, liver-kidney mineral and trace element concentrations, ADC and feed treatments in those that showed significant differences. Significance for all statistical tests was accepted at probability levels of 0.05 or less. One faecal sample for the experimental feed containing 40% mineral supplement collected on day 45 was contaminated with feed pellets and was not processed or used in any statistical analysis.

4.2.4.2 Statistical power

The statistical power of this experiment varied for each type of sample analysed, and within those samples for each element. The statistical power of each set of samples was determined *a posteriori* using the power tables and the standardised effect size index calculated from the following formula:

$$f = \sqrt{\left(\frac{k-1}{kn}\right)F} \qquad [4.4]$$

(Searcy-Bernal, 1994) where f is the standardised effect size index, k the number of treatments, n the sample size, and F the F value from an analysis of variance.

4.3 Results

4.3.1 Concentration of minerals in water

Over the course of the experiment, the mean concentration of phosphorus present in the water samples taken from the experimental system was significantly greater than those samples taken from the input water (Table 4.3). The level of phosphorus increased in the system samples after the first week and rose steadily until the last sample, although the level of phosphorus in the input water decreased throughout the entire period of the experiment (Figure 4.1).

4.3.2 Feed mineral and trace element concentrations

The experimental feeds differed significantly in mean concentrations (mg kg^{-1}) of the following supplemented elements: copper, manganese, potassium, sodium, phosphorus and zinc (Tables 4.4 and 4.5). All the mean concentrations of these elements, except sodium, were positively correlated with the percentage of mineral and trace element supplementation included in the experimental feed: copper ($r = 0.557$, $P = 0.013$, $n = 20$), manganese ($r = 0.755$, $P < 0.001$; $n = 20$), potassium ($r = 0.785$, $P < 0.001$; $n = 20$), phosphorus ($r = 0.663$, $P = 0.002$; $n = 20$) and zinc ($r = 0.602$, $P = 0.006$; $n = 20$), and sodium showed no correlation with supplement level ($r = 0.314$, $P > 0.05$; $n = 20$). The feeds did not differ significantly in the remaining supplemented elements, calcium, magnesium, iron, cobalt and selenium.

Table 4.3 Mean (±SEM, n = 6) concentrations (ppm) of minerals and trace element in experimental water taken weekly over the 60 d of the experiment

Element	Sample source		F value	P
	Input	System	(df=1)	
Al	0.141 (0.031)	0.110 (0.039)	2.35	ns
B	0.064 (0.007)	0.073 (0.010)	2.71	ns
Ca	8.451 (1.001)	9.206 (1.267)	1.31	ns
Fe	0.010 (0.004)	0.008 (0.003)	0.91	ns
Mg	2.745 (0.385)	2.867 (0.402)	0.30	ns
Mo	0.012 (0.004)	0.014 (0.006)	0.21	ns
Na	7.088 (1.043)	7.687 (1.166)	0.88	ns
P	0.220 (0.055)	0.344 (0.119)	5.33	0.44
Pb	0.034 (0.019)	0.028 (0.008)	0.56	ns
S	3.277 (0.551)	3.027 (0.544)	0.63	ns
Si	3.753 (0.648)	3.534 (0.605)	0.37	ns
Sn	0.030 (0.013)	0.032 (0.007)	0.12	ns

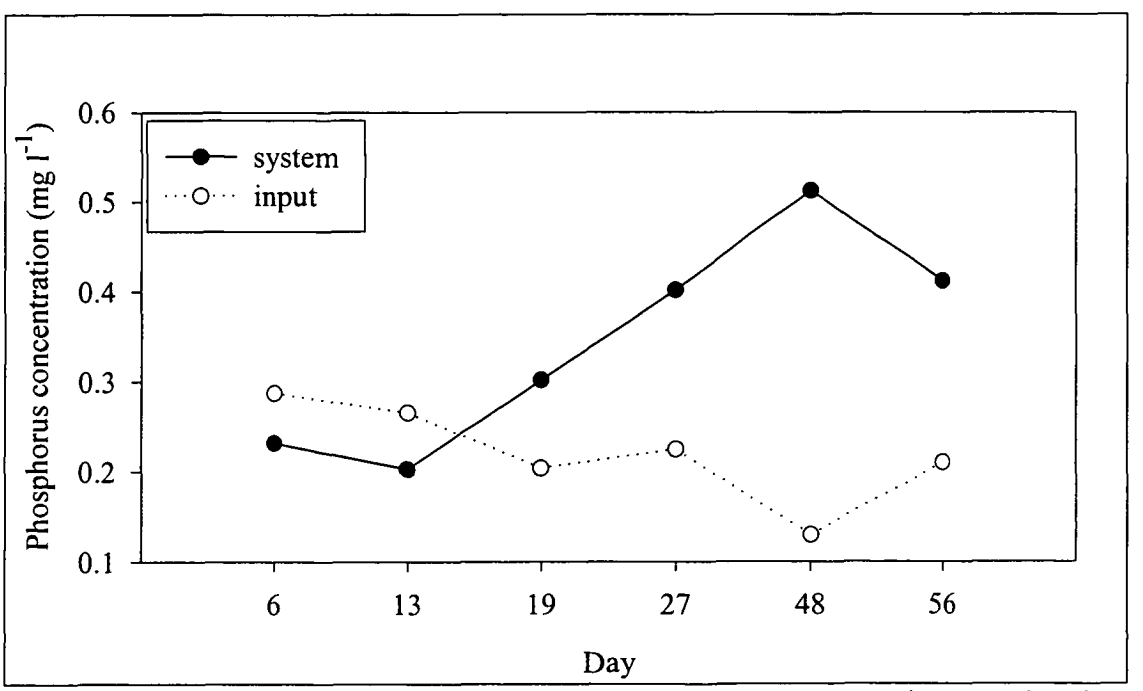

Figure 4.1 Phosphorus concentration in system and input water over time. Each point represents a single water sample.

Table 4.4 Mean mineral concentrations (mg kg^{-1}) of the experimental feeds and the percentage of that element provided by supplementation

Mineral	Supplement level				F value	*P*
	0%	20%	40%	80%	(*df*=3)	
Ca	23097.0 (6531.1)	24600.2 (4769.2) 2.3%	24375.1 (5172.6) 4.6%	25125.6 (5331.1) 8.9%	0.11	ns
Fe	226.4 (87.7)	400.1 (133.1) 2.0%	383.3 (51.3) 4.2%	350.1 (55.2) 9.2%	2.73	ns
K	8447.1^{a} (1481.4)	12491.0^{ab} (425.4) 21.6%	17017.9^{bc} (514.3) 31.6%	19705.5^{c} (6208.9) 54.7%	10.24	0.001
Mg	2516.6 (338.1)	2446.4 (123.6) 0.3%	2313.2 (79.8) 0.6%	2413.4 (53.8) 1.2%	1.11	ns
Na	13050.2^{b} (2281.5)	11022.6^{ab} (431.0) 7.1%	10596.0^{a} (431.1) 14.8%	12538.5^{ab} (585.4) 25.1%	5.19	0.012
P	18152.9^{a} (3684.8)	21085.6^{ab} (1171.4) 10.1%	23329.2^{ab} (792.1) 18.3%	25470.4^{b} (4998.2) 33.5%	4.43	0.020
S	6507.1 (1383.9)	7249.7 (314.0) 0.7%	6994.4 (247.0) 1.5%	7148.7 (210.0) 2.9%	1.06	ns

Means (±SEM, $n = 5$) with different superscripts were significantly different (Tukey's HSD).

Table 4.5 Mean trace element concentrations (mg kg^{-1}) of the experimental feeds and the percentage of that element provided by supplementation[1]

Mineral	Supplement level				F value	*P*
	0%	20%	40%	80%	(*df*=3)	
Al	14.81	13.95	12.38	10.09	0.32	ns
	(9.35)	(0.53)	(2.64)	(5.08)		
B	8.52	7.72	7.22	11.30	1.07	ns
	(5.41)	(3.27)	(4.05)	(2.60)		
Cr	7.57	2.80	3.87	4.07	0.73	ns
	(8.25)	(2.50)	(2.38)	(1.52)		
Cu	5.61^{a}	8.64^{ab}	6.59^{ab}	13.12^{b}	3.57	0.040
	(3.32)	(3.20)	(3.10)	(5.21)		
		13.9%	36.4%	36.5%		
Mn	17.52^{a}	21.90^{a}	24.19^{ab}	32.53^{b}	6.78	0.004
	(5.07)	(2.19)	(4.14)	(7.78)		
		23.8%	43.0%	64.0%		
Mo	2.37	1.61	1.90	1.48	0.23	ns
	(2.98)	(1.12)	(0.83)	(1.45)		
Ni	5.51	3.05	2.75	1.35	0.87	ns
	(7.78)	(2.57)	(2.20)	(0.61)		
Pb	3.41	0.80	5.19	4.46	0.60	ns
	(4.97)	(0.22)	(4.44)	(2.60)		
Se	3.96	5.03	4.11	6.19	0.44	ns
	(3.57)	(3.55)	(3.65)	(2.69)		
		1.2%	3.0%	3.9%		
Si	177.70	166.13	152.06	187.96	3.16	ns
	(17.87)	(23.80)	(5.46)	(4.22)		
Y	747.48	831.62	795.06	798.81	0.76	ns
	(64.17)	(85.96)	(77.23)	(98.06)		
Zn	46.37^{a}	52.55^{ab}	55.06^{ab}	58.65^{b}	3.38	0.46
	(9.83)	(1.06)	(5.11)	(5.55)		
		6.5%	13.4%	23.3%		

Means (±SEM, $n = 5$) with the same superscript were not significantly different (Tukey's HSD).

[1] Only specifically supplemented elements were assessed for percentage provided.

4.3.3 Growth performance

There were no significant differences in weight gain or specific growth rate (SGR) over the 60 days of the experiment (Table 4.6). Survival was greater than 98% for all four feed treatments. Those mortalities that occurred were the result of fish escaping from experimental tanks and not the result of any experimental treatment.

4.3.4 Apparent digestibility coefficients

There were a number of interactions between mineral supplementation and sampling day on the ADC calculated (Table 4.7). Therefore, it was not possible to determine the main effects of each factor for these elements (Zar, 1984). An interaction between feed and sample time was apparent for calcium, magnesium and manganese (Figure 4.2), which clearly showed the differences between the feed treatments increased over time. The *post-hoc* analysis (Tukey's HSD) of the calcium ADC for the combined factors (mineral supplementation*sampling day) and one-way ANOVA of each treatment factor (mineral supplementation and sampling day) revealed no significant differences. Molybdenum, phosphorus and silicon showed no differences between feed treatments over time, but there were reductions in the amount of variability in ADC calculated for the later sampling days (Figure, 4.3). The remaining elements, mostly trace elements, showed small differences in ADC over time, (Table 4.8), and potassium and selenium, differed slightly by feed treatment (Table 4.9). Interestingly nickel, an element that was not specifically supplemented, showed significant differences in ADC calculated for each feed on every sampling day (Figure 4.4).

Table 4.6 Effect of the experimental feeds on the growth performance parameters of Atlantic salmon

Parameter	Unit	Supplement level				*P*
		0%	20%	40%	80%	
Initial BW	(g)	27.0 (0.3)	27.4 (0.4)	28.2 (0.3)	27.5 (0.4)	ns
Final BW	(g)	98.7 (4.5)	102.9 (6.4)	101.3 (6.0)	103.7 (4.4)	ns
Weight gain	(g)	71.7 (6.7)	75.5 (1.2)	73.2 (3.5)	76.2 (6.0)	ns
SGR	(% d^{-1})	2.2	2.2	2.1	2.2	ns
Overall survival	(%)	100	99	99	100	ns

Each value is the mean (±SEM) of five tank replicates.

Table 4.7 Results of a two way analysis of variance (ANOVA) examining the effect of feed and sampling day on the ADC (%) calculated for minerals and trace elements

Mineral	Feed		Sampling day		Feed * Sampling day	
	F value (*df*=3)	*P*	F value (*df*=2)	*P*	F value (*df*=6)	*P*
Al	2.01	ns	5.82	0.005	0.54	ns
Ca	1.69	ns	0.08	ns	2.79	0.021
Cu	1.58	ns	0.37	ns	1.00	ns
Fe[1]	0.57	ns	1.76	ns	0.53	ns
K	26.10	<0.001	0.13	ns	0.62	ns
Mg	16.20	<0.001	0.46	ns	2.39	0.043
Mn	0.19	ns	0.74	ns	3.15	0.011
Mo	2.00	ns	29.26	<0.001	2.70	0.025
Na	1.25	ns	0.82	ns	1.57	ns
Ni	89.26	<0.001	0.07	ns	0.82	ns
P	4.16	0.010	4.69	0.013	3.80	0.004
Pb	1.62	ns	4.19	0.020	1.81	ns
S	0.46	ns	89.86	<0.001	0.45	ns
Se	0.03	0.025	3.25	0.046	0.34	ns
Si	0.18	ns	4.13	0.021	2.40	0.042
Zn	0.75	ns	6.63	0.003	1.80	ns

[1] Data for this element was arcsine transformed prior to statistical analysis.

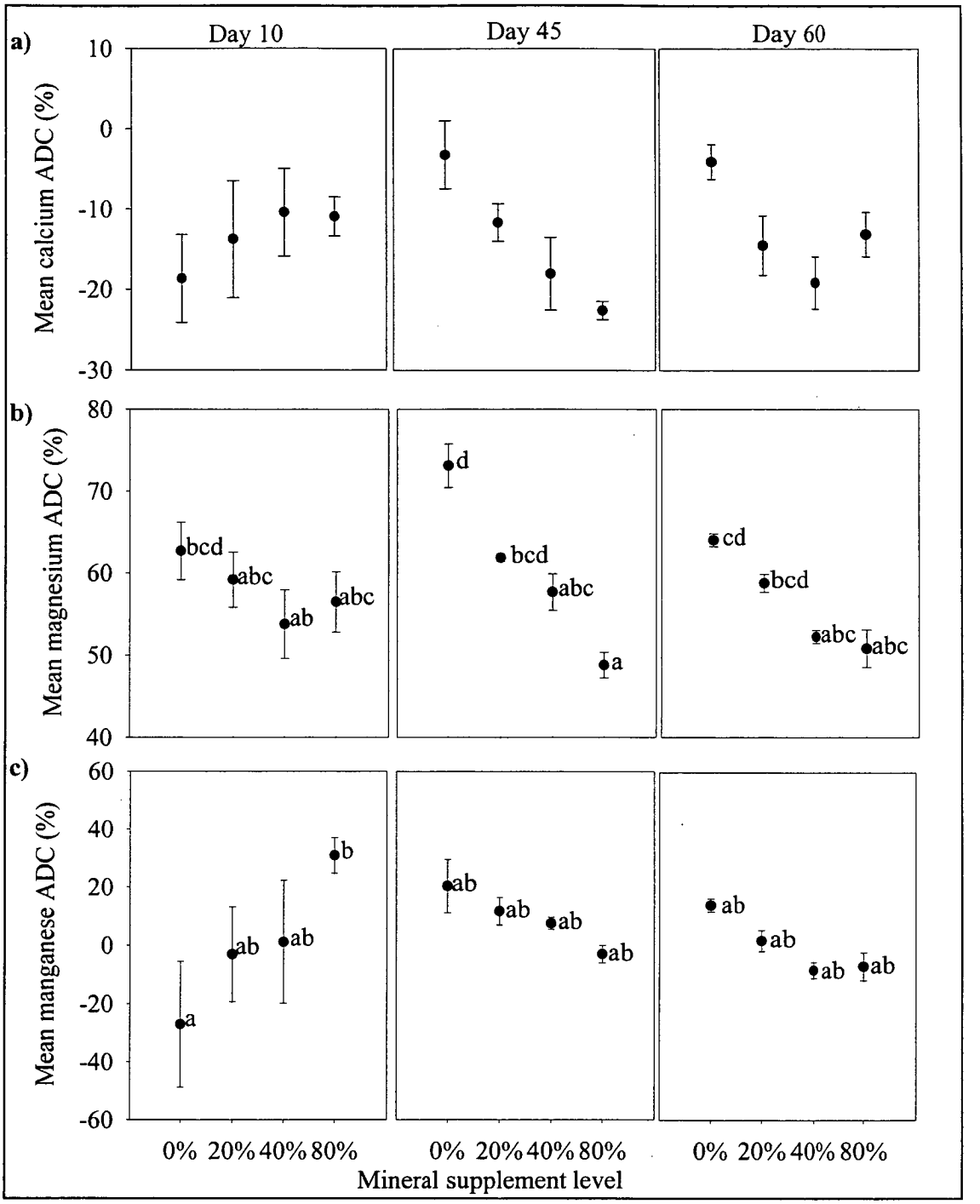

Figure 4.2 Effect of experimental feeds and sampling day on mean (±SEM, $n = 5$)

a) calcium, b) magnesium and c) manganese ADC (%) calculated from faecal samples collected on days 10, 45 and 60. Mean ADC with the same superscript were not significantly different (Tukey's HSD).

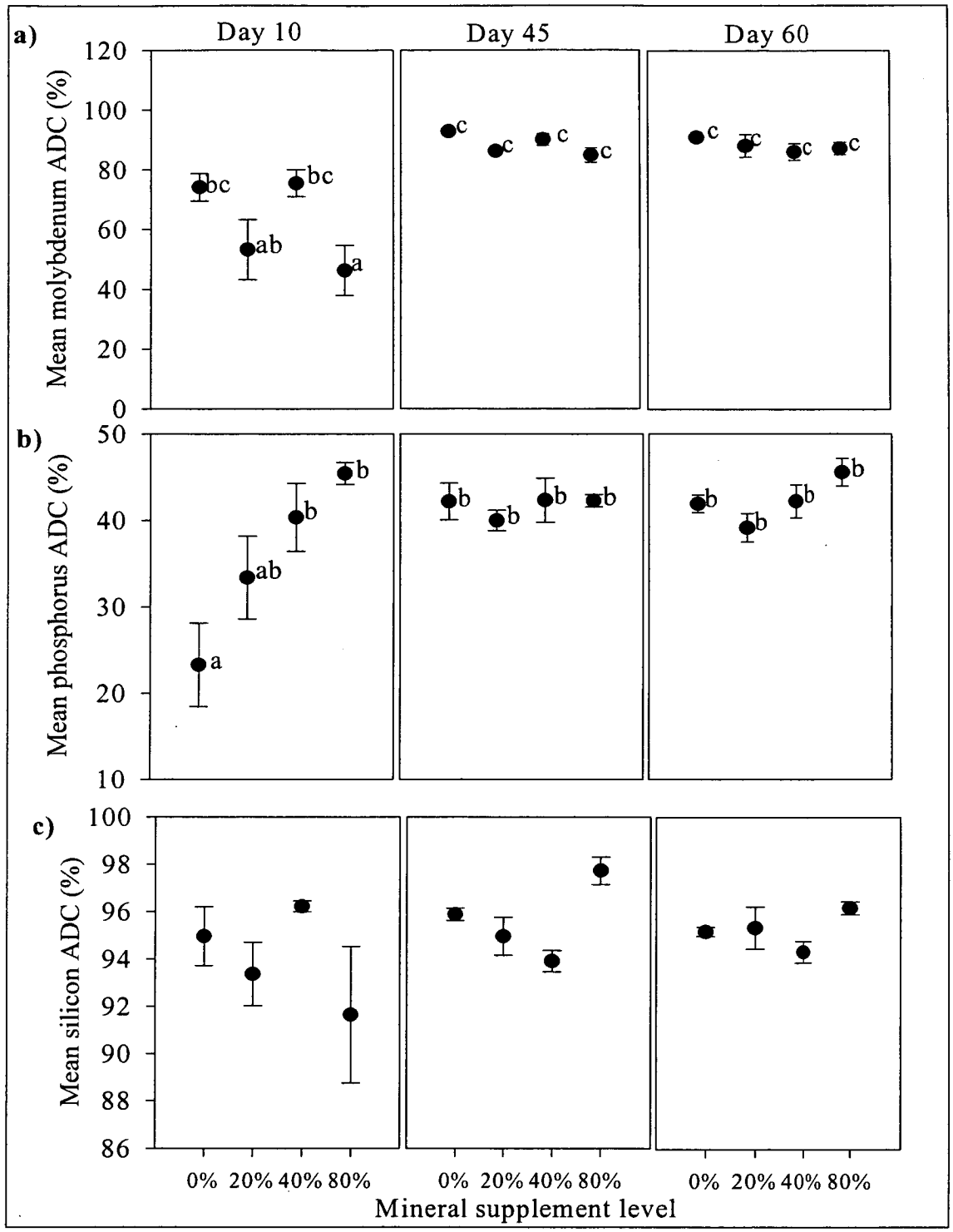

Figure 4.3 Effect of experimental feeds and sampling day on mean (±SEM, $n = 5$) a) molybdenum, b) phosphorus and c) silicon ADC (%) calculated from faecal samples collected on days 10, 45 and 60. Mean ADC with the same superscript were not significantly different (Tukey's HSD).

Table 4.8 Effect of sampling day on mean (±SEM, n = 5) ADC (%) calculated from faecal sample taken on days 10, 45 and 60.

Element	Sample day			F value	P
	10	45	60	(df=3)	
Al	-642.58[a] (538.39)	-232.87[b] (141.40)	-525.80[ab] (354.33)	5.82	0.005
Mo	62.22[a] (19.86)	88.32[b] (4.76)	87.95[b] (5.85)	29.26	<0.001
Pb	64.68[a] (72.91)	98.31[b] (5.74)	98.90[b] (4.20)	4.19	0.020
S	98.54[b] (4.24)	87.72[a] (1.85)	88.69[a] (1.45)	89.86	<0.001
Se	95.45 (8.97)	100.00 (0.00)	96.00 (4.93)	3.25	0.046
Si	94.04[ab] (3.92)	92.94[a] (1.30)	95.30[b] (1.30)	4.13	0.021
Zn	40.50[b] (12.76)	29.62[a] (10.75)	40.69[b] (9.13)	6.63	0.003

Means with the same superscript were not significantly different (Tukey's HSD).

Table 4.9 Effect of experimental feeds on mean (±SEM, n = 5) ADC (%) calculated from all faecal samples collected for each feed treatment

Element	Supplement level				F value	P
	0%	20%	40%	80%	(df=11)	
K	99.37[a] (0.18)	99.59[b] (0.11)	99.70[b] (0.05)	99.72[c] (0.10)	26.10	<0.001
Ni	89.12[c] (6.96)	71.45[b] (9.08)	64.32[b] (9.32)	24.00[a] (16.97)	89.26	<0.001
Se	97.09 (8.86)	97.10 (4.84)	96.94 (5.17)	97.53 (5.56)	0.03	0.025

Means with the same superscript were not significantly different (Tukey's HSD).

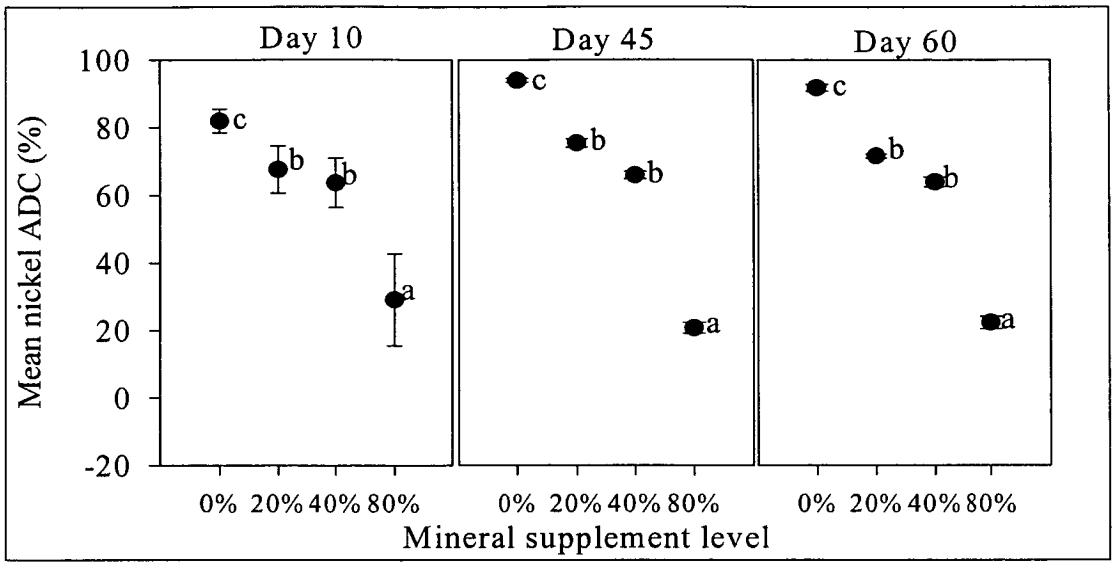

Figure 4.4 Effect of experimental feeds and sampling day on mean (±SEM, $n = 5$) nickel ADC (%) calculated from faecal samples collected on days 10, 45 and 60. Means with the same superscript were not significantly different (Tukey's HSD).

There were a number of significant correlations between ADC calculated for each element from faecal samples collected on day 60 (Table 4.10). There were highly significant ($P < 0.001$) positive correlations between ADC for copper - iron, manganese - calcium, manganese - magnesium, nickel - magnesium, zinc - iron, calcium – magnesium, sodium – sulphur, and potassium and the level of mineral supplementation. The ADC for magnesium and nickel both had highly significant ($P < 0.001$) negative correlation related to the level of mineral supplementation.

4.3.5 Concentrations of minerals and trace elements in tissue samples

Tissue samples varied in the total concentration of minerals and trace elements by tissue type. Within tissue types mineral and trace element concentrations varied over time. Tissue concentrations below the level of detection for an element were not reported. The level of detection was based on internal quality control samples used at the time of analysis and results from dogfish muscle (DORM-2) results that were processed and analysed at the same time as the tissue samples.

4.3.5.1 Blood samples

There was a significant interaction ($F = 3.023$, $P = 0.048$) between sampling day and experimental feed on mean blood sodium concentrations (Figure 4.5a). However, there was no significant correlation between mineral supplement level and mean blood sodium concentrations ($r = -0.191$, $P = 0.271$, $n = 35$). Mean concentrations of

Table 4.10 Correlation coefficients (Pearson's, $n = 20$) between mineral and trace element ADC and the mineral supplementation level and mineral concentrations

ADC	Mineral supp.	[Ca]	[Fe]	[K]	[Mg]	[Na]	[P]	[S]
B	0.084	0.538*	0.552*	-0.025	0.227	0.282	0.487*	0.428
Cu	-0.019	0.418	0.918***	-0.141	0.191	0.101	0.260	0.278
Mn	-0.651**	0.693***	0.511*	-0.584**	0.817***	-0.277	0.081	-0.227
Ni	-0.985***	0.305	0.235	-0.660**	0.774***	-0.622**	-0.415	-0.668**
Pb	0.265	-0.406	0.007	0.482*	-0.293	0.154	-0.137	0.170
Si	0.260	0.354	0.058	0.159	0.012	0.497*	0.418	0.505*
Zn	-0.179	0.477*	0.835***	-0.101	0.326	-0.071	0.318	0.169
Ca	-0.338	1.000	0.456*	-0.363	0.727***	0.124	0.586**	0.236
Fe	-0.296	0.456*	1.000	-0.382	0.389	-0.145	0.103	0.019
K	0.688***	-0.363	-0.382	1.000	-0.536*	0.597**	0.290	0.615**
Mg	-0.813***	0.727***	0.389	-0.536*	1.000	-0.161	-0.013	-0.186
Na	0.544*	0.124	-0.145	0.597**	-0.161	1.000	0.357	0.928***
P	0.452*	0.586**	0.103	0.290	-0.013	0.357	1.000	0.543*
S	0.603**	0.236	0.019	0.615**	-0.186	0.928***	0.543*	1.000

* Significant at the 0.05 level; ** significant at the 0.01 level; ***significant at greater than the 0.001 level

iron, potassium, phosphorus, sulphur and zinc in the blood rose significantly over time, but showed no significant differences between feed treatments (Figures 4.5 and 4.6). Mean blood silicon concentrations fell significantly over time, and mean calcium and magnesium concentrations did not differ significantly (Figure 4.7). The following trace elements had concentrations below confidence levels and were not reported: aluminium, arsenic, boron, chromium, copper, manganese, molybdenum, nickel, lead, selenium, and yttrium.

4.3.5.2 Muscle samples

There were significant differences in minerals and trace elements concentrations in muscle tissue over the period of the experiment (Table 4.11). There were no significant correlations between supplementation level and muscle tissue concentrations. The concentrations of boron, magnesium, and sulphur rose while potassium, sodium and phosphorus levels fell between the two sampling periods.

4.3.5.3 Combined liver-kidney samples

There were many significant differences in mineral concentrations in the combined liver-kidney samples over time (Table 4.12). Of the supplemented elements, iron, potassium and zinc concentrations increased. Whereas, the concentrations of and arsenic, calcium, magnesium, manganese, sodium, phosphorus, lead and decreased. Only copper, selenium and vanadium showed significantly different concentrations

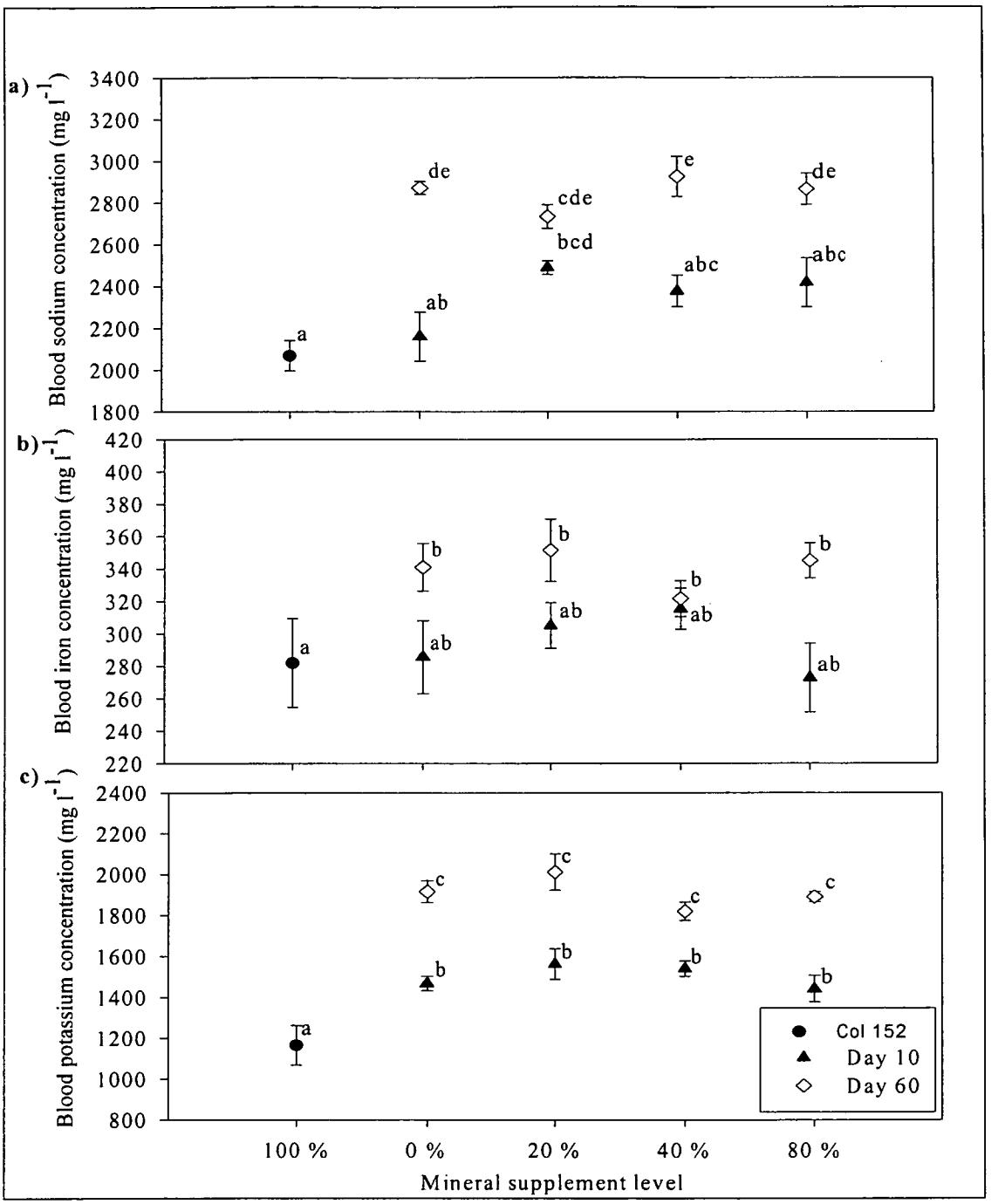

Figure 4.5 Effect of experimental feeds and sampling day on mean (±SEM, $n = 5$) concentrations of a) sodium ($F = 3.02$, $P = 0.048$), b) iron ($F = 13.65$, $P = 0.001$) and c) potassium ($F = 81.11$, $P < 0.001$) in the blood of Atlantic salmon. There was a significant interaction between sampling day and experimental feed for sodium concentrations. Means with the same superscripts were not significantly different (Tukey's HSD).

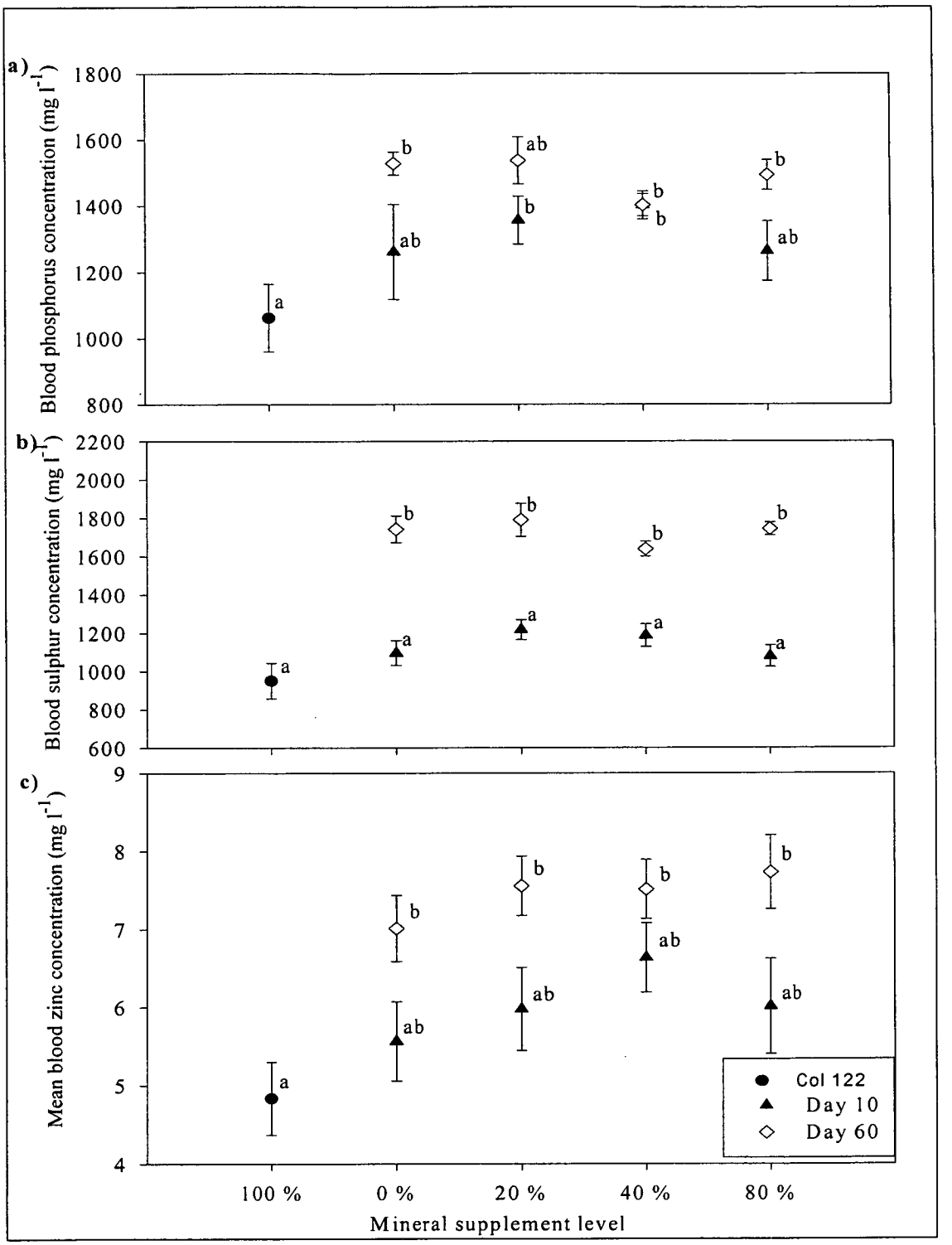

Figure 4.6 Effect of sampling day on mean (±SEM, $n = 5$) concentrations of blood a) phosphorus ($F = 12.02$, $P = 0.002$), b) sulphur ($F = 152.67$, $P < 0.001$) and c) zinc ($F = 17.71$, $P < 0.001$). Means with the same superscripts were not significantly different (Tukey's HSD).

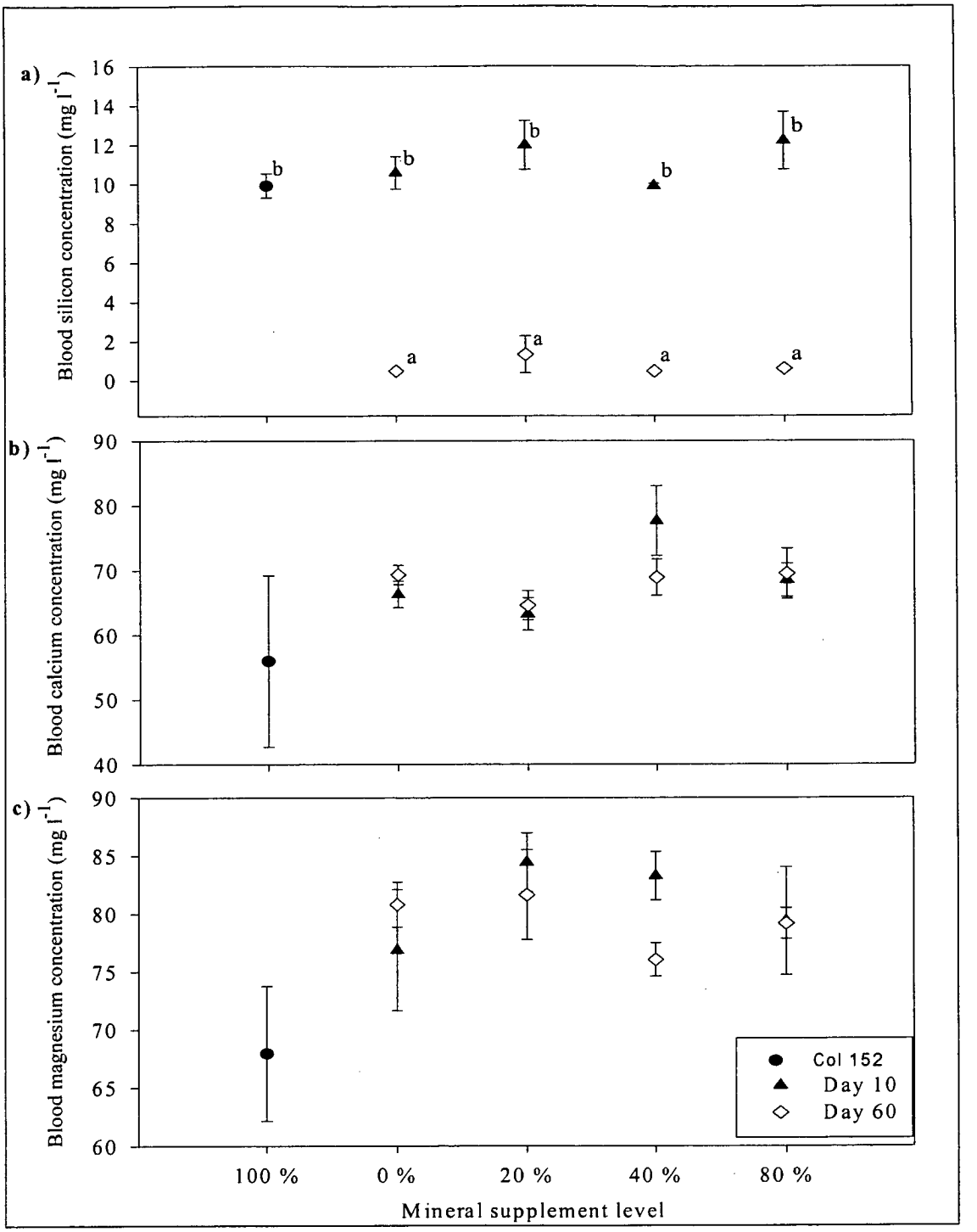

Figure 4.7 Effect of sampling day on mean (±SEM, $n = 5$) concentrations of blood a) silicon ($F = 453.44$, $P < 0.001$), b) calcium and c) magnesium. There were no significant effects of sampling day or experimental feed on mean blood calcium or magnesium concentrations. Means with the same superscripts were not significantly different (Tukey's HSD).

Table 4.11 Effect of sampling day on mean (±SEM, $n = 20$) muscle mineral and trace element concentrations (mg kg^{-1})

Mineral	Sample Day		F value	*P*
	10	60	(*df*=1)	
Al	20.78 (33.76)	6.59 (3.60)	3.49	ns
B	0.46 (0.45)	0.85 (0.51)	6.21	0.017
Ca	116.66 (24.46)	123.79 (94.91)	0.10	ns
Cd	0.58 (0.53)	0.24 (0.22)	6.93	0.012
Cr	1.67 (0.56)	0.59 (0.61)	34.17	<0.001
Cu	4.61 (2.18)	2.24 (1.12)	18.71	<0.001
Fe	2.64 (1.04)	2.23 (0.69)	1.67	ns
K	4679.31 (182.75)	4007.22 (172.36)	143.08	<0.001
Mg	289.92 (11.08)	308.52 (24.67)	9.20	0.004
Na	216.72 (24.81)	196.81 (18.81)	8.27	0.007
Ni	2.70 (1.04)	1.08 (0.87)	28.43	<0.001
P	2899.99 (86.39)	2642.63 (110.91)	67.76	<0.001
S	1681.94 (42.81)	1770.93 (44.82)	41.86	<0.001
Se	2.28 (1.93)	1.36 (0.89)	3.74	ns
Zn	3.84 (2.18)	2.92 (1.99)	1.97	ns

Table 4.12 Effect of sampling day on mean (±SEM, $n = 20$) liver-kidney sample mineral and trace element concentrations (mg kg^{-1})

Mineral	Sample Day		F value	*P*
	10	60	(*df*=7)	
Al	23.01 (13.59)	24.25 (7.50)	0.476	ns
As	5.31 (2.39)	1.27 (1.46)	30.94	<0.001
B	32.01 (5.53)	165.75 (29.67)	710.10	<0.001
Ca	1072.36 (305.72)	556.76 (185.50)	41.58	<0.001
Fe	477.60 (68.33)	596.93 (85.58)	23.75	<0.001
K	14773.70 (516.10)	16708.48 (1909.43)	19.14	<0.001
Mg	995.07 (71.33)	743.26 (89.41)	96.94	<0.001
Mn	6.69 (1.02)	3.95 (0.73)	78.15	<0.001
Na	5663.69 (294.67)	5219.20 (540.22)	10.44	0.003
Ni	0.56 (0.75)	0.77 (0.72)	36.95	<0.001
P	16683.34 (532.40)	15709.79 (1863.29)	5.05	0.031
Pb	3.54 (1.59)	0.73 (0.73)	50.38	<0.001
S	10953.99 (371.38)	11089.75 (1229.75)	0.22	ns
Si	3.36 (3.57)	14.34 (2.35)	43.66	<0.001
Zn	77.57 (3.45)	105.30 (9.50)	76.81	<0.001

between the four feed treatment (Figure 4.8), and only copper concentrations were significantly correlated with supplement level ($r = 0.421$, $P = 0.007$, $n = 40$).

4.4 Discussion

4.4.1 Effect of mineral supplementation on feed mineral concentrations

The lack of significant differences for some supplemented minerals in the feeds indicated that the variability of minerals in the major ingredients used to create the feed, fish meal and wheat flour, was greater than supplementation levels. The variability is likely to be even greater when more than one batch of fish meal or wheat flour is used to make a feed. In the case of fish meal, the type and source of fish used to produce the meal and manufacturing and processing effects the mineral availability of the final product (Watanabe et al., 1988). In the present study the variability was not the same for all the nutrients supplemented, which was evident when the concentration of a nutrient provided by the supplement was compared to the concentration of that element in the feed. Supplementation in the feed with the highest inclusion level (80%) accounted for only 1%, 3% and 8% of magnesium, selenium and calcium respectively, but provided 54% and 63% of potassium and manganese (see Tables 4.4 and 4.5); additionally, although cobalt (cobalt sulphate) was specifically supplemented in the feed, the analytical procedures were unable to detect this element in any feed samples. This highlights the need to identify the effect of common feed ingredients, such as fish meal and wheat flour, in mineral and trace element nutrition experiments. These common ingredients form the base of many experimental feeds and commercial products, and as such will factor in other mineral

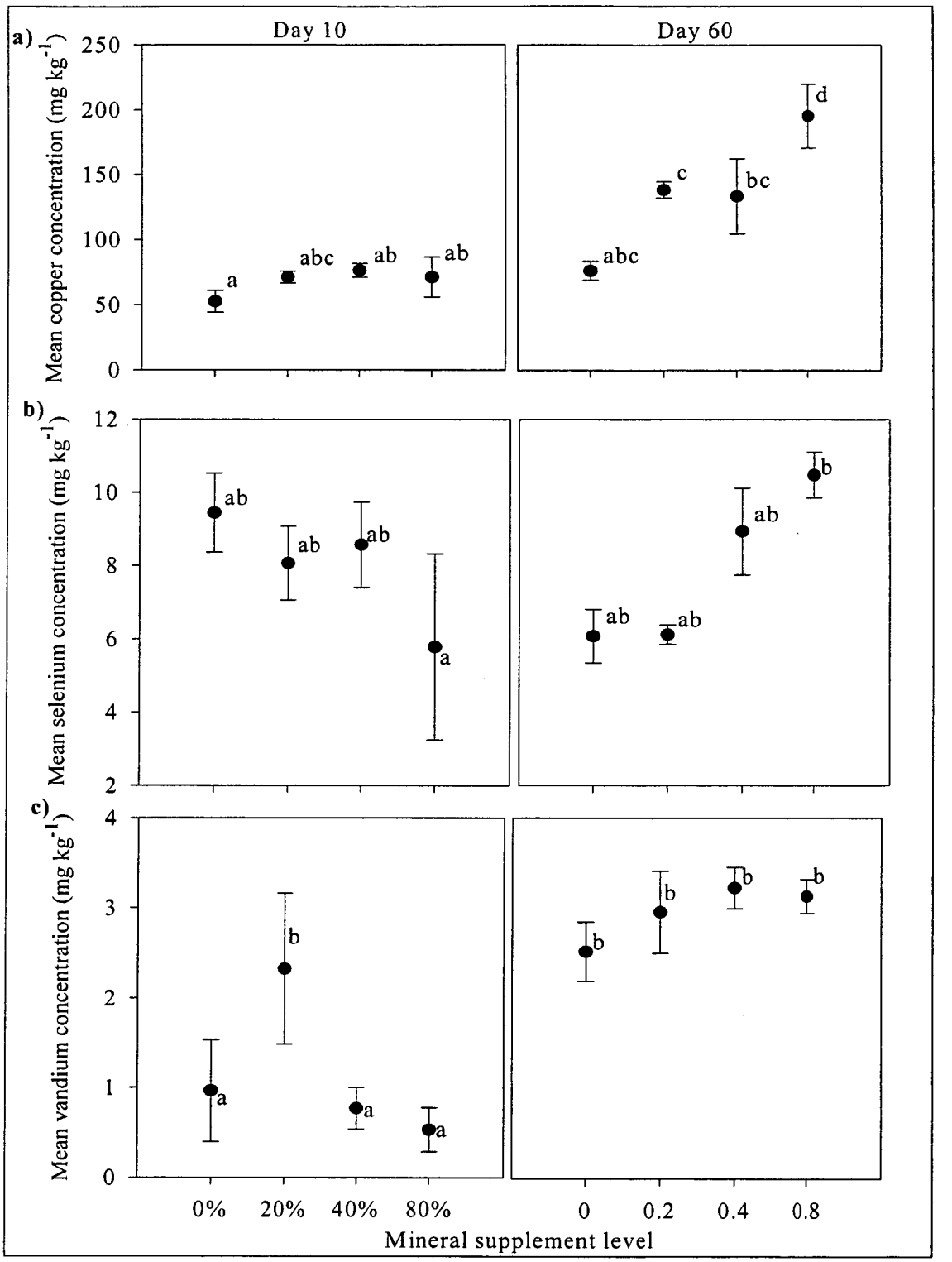

Figure 4.8 Effect of experimental feeds and sampling day on mean (±SEM, $n = 5$) concentrations (mg kg^{-1}) of a) copper, b) selenium and c) vanadium concentration in liver-kidney samples. Means with the same superscript were not significantly different (Tukey's HSD).

nutrition research (Watanabe et al., 1988; Hardy, 1999; Tisdell, 1999; Hardy & Tacon, 2001). The results from this study identify the need to focus on minerals, such as potassium and manganese, which may be limited in ingredients when conducting nutrition research. Additionally, variability in the mineral and trace element content of ingredients will vary regionally, requiring additional analyses to identify these differences. The present study provided a better understanding of the interaction between minerals present in a feed composed of common, commercial ingredients and identified the effects and interactions between supplemental minerals and trace elements on ADC and bioavailability in Atlantic salmon.

4.4.2 Effect of mineral supplementation on growth

Poor growth is the most often cited symptom of mineral and trace element deficiency in fish (Watanabe et al., 1988; Lorentzen et al., 1996; Watanabe et al., 1997; Maage et al., 2000; Lall, 2002). However, in the present experiment there were no significant differences in growth parameters between the experimental feeds, and the results were comparable to those obtained in Atlantic salmon growth experiments under similar conditions (Carter & Hauler, 2000). Additionally, no fish in the experiment exhibited any gross abnormalities, such as deformed heads, dwarfism (Watanabe et al., 1997) or cataracts (Ketola, 1979; Satoh et al., 1983) commonly associated with mineral and trace element deficiencies, although the experiment was conducted for a relatively short period of time and conditions resulting from trace element and mineral deficiencies have only been found in long term (60-93 week) experiments (Satoh et

al., 1983; Yamamoto et al., 1983). Observations based on growth suggest even the feed lacking mineral and trace element supplements provided sufficient amounts of all the required nutrients, and supplementation provided no benefit to growth over the 60 days of the experiment.

4.4.3 Apparent digestibility coefficients

ADC calculated for calcium, magnesium, manganese, molybdenum, phosphorus and silicon displayed significant interactions between feed treatment and sampling time. There appeared to be a trend related to the level of mineral supplementation and magnesium, manganese and calcium ADC. It is possible that over time or with greater differences in supplementation these trends may have become more pronounced. These interactions can be attributed to the effect of nutrient stores within the body, resulting excretion and interactions with other supplemented minerals or trace elements. There were a number of elements that showed highly significant correlations, both positive and negative, with other elements and confirmed strong a interactions between these elements. Mineral supplementation level and phosphorus ADC values had significant positive correlations, confirming suggestions that dietary calcium could be used to predict phosphorus availability without the need for controlled studies (Riche & Brown, 1996). In the case of nickel, Ptashynski and Klaverkamp (2002) provided evidence for a protective feedback mechanism controlling the uptake of nickel in lake whitefish (*Coregonus clupeaformis*), a similar mechanism may be present in salmonids. Alternatively, there may be competition for the active sites of absorption with other supplemented elements, as evidenced by the

negative correlation coefficient with the mineral supplementation level. Similarly, Berntssen et al. (1999) found significant reductions in digestibility of copper when present in feed in elevated levels, indicating a strong regulatory mechanism for the uptake of this element.

Most ADC values did not differ significantly by feed treatment, but differed according to sampling day. The effect of sampling day may have resulted from a number of factors. The demands of rapid growth resulting in changes to the digestive tract, providing more surface area for the site of uptake mechanisms or increased retention of these elements could account for changes in ADC over time. Hillestad et al. (1999) identified lower ADC for phosphorus and nitrogen ADC estimates in smaller fish (0.5 kg) than larger fish (1.0 kg), kept under similar conditions, but were unable to identify any reasons for these differences. Again, increased nutrient interaction and competition for active sites of absorption, as well as increases in faecal excretion of excess minerals are likely causes for these significant changes in nutrient ADC.

4.4.4 Mineral interactions and ADC

The concentration of dietary minerals and trace elements may affect the digestibility of other elements. The analysis of the correlations between ADC values and the concentrations of elements in the tissues sampled revealed a number of significant correlations. These correlations were associated with supplemented minerals and those trace elements that produced significant effects on the tissues sampled: copper, calcium, phosphorus, sulphur, iron, and magnesium. Calcium and phosphorus ADC

were most often correlated to each other and other ADC values. In a number of instances the concentration of minerals and trace elements were highly positively correlated with the digestibility of other elements although there weren't in all cases significant correlations between those minerals and the level of mineral supplementation (Table 4.10). The ADC of copper and zinc were highly correlated with the concentration of iron in the feed. Additionally, the ADC of magnesium and manganese were highly correlated with the concentration of calcium present. This suggests that a number of possible biologic reasons for these correlations: common transport systems are operating for these elements; the transport system for these elements linked; the elements may be present in organic compounds in the feed ingredients that are more readily digestible than the inorganic supplements. There were few correlations between concentrations of the other elements in tissue samples. There were more correlations within tissue samples than between samples, and this may reflect the mineral retention capacity of each tissue. In the case of phosphorus, there were no significant differences in the concentration of phosphorus in the blood or liver-kidney samples and a small decrease in the muscle content, and there were stable ADC for phosphorus at days 45 and 60. The lack of significant correlations between ADC and the mineral and trace element content of the tissue samples may be related to the length of time required to alter homeostatic and/or hormonal controls involved in the uptake and retention of minerals. The high growth exhibited by fish of this size may limit the effect of feedback systems at such high levels of feeding.

The concentrations of nutrients in the water entering the experimental system and that within the system differed only with respect to phosphorus. It is most likely that this phosphorus has been excreted in the urine and may represent some soluble forms of

the elements present in the faeces. The lack of significant differences in phosphorus ADC over time, but not between feed treatments suggested that phosphorus originated from urinary excretion.

The ADC data from the present experiment reinforces suggestions made by Hillestad, et al. (1999) that fixed digestibility coefficients should not be used, particularly for minerals and trace elements. Changes in the level of mineral and trace element supplementation and altering feed formulations alter the inherent mineral content of a feed, and resulted in significant changes in the ADC of minerals and trace elements. Experimental feeds composed of purified or semi-purified ingredients may not accurately identify the effect of formulation changes on mineral and trace element ADC for ingredients that are then incorporated into feeds composed of common, commercial ingredients. The mineral and trace element digestibility and the variability of major feed ingredients used for feed formulations should be assessed as in the current experiment, with the graded addition of new ingredients or supplements to a reference feed. The ADC values should be calculated from a number of faecal collections taken over a period of time to indicate the initial digestibility, and possible digestibility trend for an ingredient or a feed. These ingredient ADC determinations should be conducted in fish of the size the feed is intended for, to limit any variability resulting from differences sizes classes and with time.

4.4.5 Concentrations of minerals and trace element in tissue samples

Tissue samples (blood, muscle and liver-kidney) were assessed for mineral and trace element concentrations to ascertain the effect of mineral supplementation on the mineral nutritional status of rapidly growing Atlantic salmon. The information provided by the present experiment establishes base levels for the reference feed and tissue samples from Atlantic salmon fed this feed (Table 4.13), and provide a reference for predicting the effect of minerals supplementation and further research using feeds composed of the same ingredients when looking "novel" ingredients. The concentrations of trace elements in specific tissues, such as selenium and copper in liver-kidney samples, provided an estimate of the effect of dietary mineral supplementation on homeostatic controls of the uptake, transport and storage of several elements. The blood and muscle samples indicated that there are changes in mineral and trace element concentrations in these tissues over time, but these changes did not correlate specifically with the feed treatments. This may suggest that the correlation between ADC and the concentration of minerals and trace element in tissue samples is limited, when looking at relatively slight differences in mineral and trace elements in feed. The levels of supplementation had little effect on the concentrations of some nutrients found in tissue samples, suggesting that there was no effect in those tissues, or that changes in these control systems occur over longer periods of time. Correlations between the ADC of trace elements such as boron, and silicon, while statistically significant for a number of elements had correlations with an r value less than 0.650, therefore, only those elements which showed correlation coefficients greater with $r > 0.650$ were considered to be biologically significant.

Table 4.13 Mean (±SEM) feed mineral and trace element concentrations, ADC (%) and mineral and trace element concentrations in Atlantic salmon blood, muscle and liver-kidney samples

Element	Feed	Water	ADC range	Blood	Muscle	Liver-kidney
Ca	24362.79 (4995.88)	8.83 (1.16)	-35.9 - 9.6	67.34 (9.06)	120.32 (68.50)	814.56 (361.20)
Fe	352.65 (100.84)	0.01 (0.00)	-162.7 - 55.3	319.27 (40.06)	2.41 (0.89)	537.26 (97.43)
K	14729.51 (5246.61)	0.00 (0.00)	98.9 - 99.9	1706.14 (282.88)	4343.17 (382.76)	15741.09 (1692.87)
Mg	2417.47 (173.09)	2.81 (0.38)	43.8 - 79.0	78.97 (6.92)	299.35 (21.04)	869.16 (150.44)
Na	11736.16 (1446.19)	7.39 (1.10)	82.9 - 97.2	2614.51 (328.03)	206.81 (23.98)	5441.44 (484.91)
P	22212.55 (3945.72)	0.28 (0.11)	10.6 - 50.6	1395.58 (183.34)	2772.02 (163.70)	16196.57 (1439.63)
S	6999.65 (664.81)	3.15 (0.54)	82.7 - 100.0	1461.68 (340.59)	1726.10 (62.72)	11021.87 (899.26)
Al	12.74 (5.49)	0.13 (0.04)	-1829.3 - -15.7		13.68 (24.76)	24.47 (13.31)
B	8.75 (3.92)	0.07 (0.01)	-665.4 - 81.1		0.62 (0.58)	93.07 (63.47)
Cr	4.44 (4.21)	0.00 (0.00)			1.12 (0.80)	
Cu	8.64 (4.59)	0.00 (0.00)	-57.3 - 89.9		3.40 (2.13)	100.23 (52.84)
Mn	24.38 (7.30)	0.00 (0.00)	-104.2 - 51.7			5.37 (1.63)
Mo	1.81 (1.61)	0.01 (0.01)	16.0 - 99.7			
Ni	2.91 (3.50)	0.00 (0.00)	-2.1 - 95.2		1.62 (1.81)	0.52 (0.95)
Pb	3.83 (3.69)	0.03 (0.01)	-213.6 - 100.0			1.55 (3.05)
Se	4.87 (3.21)	0.00 (0.01)	66.5 - 100.0	0.60 (0.64)	1.41 (2.75)	7.85 (2.88)
Si	170.35 (19.09)	3.64 (0.61)	81.1 - 98.6	5.09 (5.30)		9.53 (9.31)
V		0.00 (0.00)			0.49 (0.82)	1.90 (1.70)
Y	795.65 (81.66)	0.00 (0.00)	n/a			
Zn	53.51 (6.97)	0.00 (0.00)	4.9 - 55.9	6.75 (1.24)	2.44 (4.59)	88.89 (14.02)

The results obtained in the present experiment confirm results obtained in similar work conducted on rainbow trout. The use of whole blood samples may not be ideal for measuring the uptake of minerals and trace elements, but there were significant differences in the mineral content of blood over 60 days. Sugiura et al.(1998) used blood plasma and scales to measure the effect of dietary supplements on calcium, phosphorus and potassium. However, these samples had been dried and ashed in a furnace, so we were not able to directly compare the results obtained in the present experiment, as wet digestion was chosen to prevent the loss of trace elements that could arise from ashing samples in a furnace (Vandecasteele & Block, 1993). Gomes and Kaushik (1993) reported no significant differences in plasma concentrations of zinc, calcium or magnesium, when replacing inorganic zinc with zinc-methionine in rainbow trout. Maage et al. (2001) also found no significant differences in plasma zinc when investigating the effects of various forms of dietary zinc supplement. Sugiura et al. (2000b) report that urinary measurements of phosphorus proved more useful than blood analysis when determining the response of rainbow trout to dietary phosphorus. The iron concentrations of the liver-kidney samples were 5 times greater than those reported in Atlantic salmon by Standal et al. (1999) in similar sized fish. Andersen et al. (1997) reported liver iron concentrations of 188 mg kg^{-1}, which when combined with iron from the kidney would likely have been close the value reported in this investigation. Baeverfjord et al.(1998) reported muscle concentrations of 131 mg kg-1 of calcium, 3,091 mg kg^{-1} of phosphorus, and 414 mg kg^{-1} of magnesium, when investigating the effects of feeding low phosphorus feeds to Atlantic salmon. Muscle tissue samples were analysed for minerals and trace element from day 10 onward instead of day 0 to minimise the effect of fasting (El-Mowafi et al., 1997). Vertebrae are also used to measure the effect of supplementation of

minerals (El-Mowafi et al., 1997; El-Mowafi & Maage, 1998). Lorentzen et al. (1996) found that liver manganese concentration did not respond to supplementation and stayed within a narrow range. El-Mowafi et al.(1997) reported significant decreases in liver concentrations of phosphorus, zinc and copper in fasting Atlantic salmon, with pre-fasting levels similar to those reported here. Rønsholdt (1995) identified significant differences in the phosphorus concentration of rainbow trout related to the fat content and body size of the fish. Tissues samples are often taken to measure the effect of heavy metals on finfish, and Ptashynski and Klaverkamp (2002) reported that the kidneys are a good tissue to sample for assessing the toxicology of nickel in lake whitefish (*Coregonus clupeaformis*), and that exposure to concentrations of nickel in feed, many times greater than those in the feed in this experiment, altered copper and zinc concentrations in other tissues. The authors also identified the detection limits of nickel, copper and zinc in many tissues, provided by graphite furnace atomic absorption spectrophotometry and flame atomic absorption spectrophotometry, which were less accurate those provided by the ICP-OES analysis used in the present study.

4.4.6 Experimental approach

4.4.6.1 Analytical methods

The analytical methods used in this experiment, ICP-OES analysis of samples decomposed in open vessels with concentrated nitric acid, were sufficient for accurately determining the concentrations of all minerals and many trace elements in the samples used. It was easier to decompose blood, muscle, and liver-kidney

samples than feed and faecal samples, which often required longer decomposition with additional acid and contained higher proportions of material that does not decompose fully. Stronger decomposition methods are required to process these samples, and longer decompositions could lead to the loss of volatile forms of some minerals (such as selenium) and trace elements reducing analytical precision and accuracy. It is possible that ICP-OES is not the ideal choice for analysing trace and ultra-trace elements (As, Cd, Cr, Co, Mo, Se, Sn, and V) in some types of sample. Mineral variability, from elements such as calcium, phosphorus and potassium, can create overlaps in spectral emission wavelengths resulting in difficulties when attempting to examine trace elements. It is possible to use alternative methods of analysis, such as fluorescence spectrophotometry (Koh & Benson, 1983) or other means of decomposition and detection (Vandecasteele & Block, 1993), to better determine the concentrations and bioavailability of these trace elements.

Significant rises in the concentration of silicon in the liver-kidney samples may reflect retention of silicon ingested from the water supply, which showed relatively high levels of this element. The same could be said for the increases in boron concentrations in the muscle and liver-kidney tissue samples. It is possible that the increases in boron in tissue samples were due to increases in other elements within these samples causing interactions with the surface of the decomposition vessels, which were composed of boro-silicate glass. The sample blanks should have removed the effect of boron generated in the sample from the decomposition process, and all tissue samples were processed together limiting the effect of decomposition on the results.

4.4.6.2 Statistical power and effect size

Considering the expense of lengthy aquaculture nutrition trials it is essential that experiments are designed carefully (Shearer, 2000; Ling & Cotter, 2003), and consider statistical power and the effect size of treatments. Statistical power is a measure of the confidence that an experiment will yield significant results when a treatment effect exists, and effect size is the amount of variance in a measurement that is directly attributable to the experimental treatments employed. The present experiment provided an opportunity to examine the statistical power resulting from various components of this experiment, and an estimation of the effect size (Searcy-Bernal, 1994) of the mineral supplementation treatments, derived from the ANOVA results, on the mineral concentrations of the samples taken and ADC (Table 4.14).

There were differences in statistical power and effect sizes for each element depended on the type of sample analysed. In general terms, the statistical power of analysing tissue samples (blood, muscle and liver-kidney) for changes in the concentrations of elements as a result of supplementation was greater than that provided by feed, faeces

Table 4.14 The statistical power, the percentage probability of not committing a type II error (accepting a null hypothesis) for the given ANOVAs for each sample type, provided by the experimental design and analytical procedures for determining significant differences between the elemental content of the experimental feeds, fish tissues samples, faecal samples, ADC grouped by feed treatment and ADC grouped by sampling day

Element	Sample types used for ANOVA						
	Feed	Blood	Muscle	Liver and kidney	Faeces	ADC * mineral supplementation level	ADC * Sampling day
Al	8	n/a	67	n/a	7	44	88
As	n/a	n/a	n/a	<99	7	n/a	n/a
B	25	n/a	90	<99	9	n/a	n/a
Ca	6	33	7	<99	79	38	7
Cr	17	n/a	<99	n/a	7	n/a	n/a
Cu	68	n/a	<99	n/a	7	36	9
Fe	57	58	37	<99	7	13	39
K	<99	<99	<99	<99	11	<99	6
Mg	25	43	<99	<99	78	<99	11
Mn	93	n/a	n/a	<99	79	7	17
Mo	7	n/a	n/a	n/a	6	44	<99
Na	84	<99	98	<99	8	29	19
Ni	20	n/a	<99	n/a	54	<99	7
P	77	80	<99	83	72	74	80
Pb	14	n/a	n/a	<99	14	36	75
S	24	<99	<99	7	8	11	<99
Se	11	<99	70	n/a	8	9	64
Si	62	87	n/a	<99	10	7	74
Zn	65	<99	43	<99	41	17	92

n/a = not applicable

or ADC. These differences between sample types reflect the mean concentrations of the element in each sample, and possibly the effect of sample processing. For elements like zinc, copper and selenium experiments should be designed using tissue samples which provide strong statistical power. Future experiments should consider the effects of the relatively small differences in treatments on the statistical power of experiments determining trace element ADC (Searcy-Bernal, 1994). Recent work on the number of fish and replicates required to conduct nutritional experiments in aquaculture (Ling & Cotter, 2003) suggest that the number of tanks and replicates used was appropriate but the low effect sizes for some elements required more consideration when formulating experimental feeds. Semi-purified ingredients may be required to limit the effect of mineral variability in common ingredients on the effect size of feed treatments in future work. This does not alleviate the previously discussed need to examine mineral nutrition using practical feed ingredients.

4.5 Conclusions

In conclusion, while significant differences in mineral and trace element concentrations were found in the various tissues sampled, these did not always correlate to the significantly different ADC observed over the same period for those elements. Significant correlations between the ADC calculated for supplemented minerals and trace elements may allow us to estimate the digestion of others, if other means of analysis are used that do not determine multiple concentrations. In only a few cases was mineral supplementation reflected in retention differences in blood, muscle and liver and kidney samples. However, tissue samples provided much

stronger statistical power for identifying small differences between elements such as: copper, iron, selenium, and zinc. The methods of analysis used in this experiment did not effectively determine the ADC of trace elements such as cadmium, cobalt, copper, manganese, molybdenum, selenium, and tin. These elements will need to be targeted specifically when working with common feeds ingredients with high levels of mineral and trace element variability. The data compiled for trace element and mineral ADC and the tissue concentrations of the fish fed this reference feed will comprise a useful data set for comparison with other experimental feeds in future nutrition research, when using a control feed composed of these common ingredients and mineral and trace element supplementation levels. Particularly when plateaus in tissue concentrations were identified.

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Chapter 5

The effect of citric acid on mineral digestibility and blood mineral concentrations in juvenile Atlantic salmon (*Salmo salar* L.) fed a fish meal based feed with or without mineral supplementation

Abstract

Fish meal based feeds containing graded additions of mineral supplements and either 4% or 8% citric acid were fed to Atlantic salmon (*Salmo salar* L.) parr. Blood and faecal samples were collected on day 1 and day 14 and analysed for minerals and trace elements and apparent digestibility coefficients (ADC) calculated. Citric acid supplementation improved the ADC of potassium and sulphur, and when combined with mineral supplements improved the ADC of aluminium, chromium, molybdenum, nickel, calcium, magnesium and phosphorus. The overall ADC, regardless of mineral supplementation, of aluminium, boron, silicon, zinc and phosphorus were significantly higher and nickel, potassium and sodium significantly lower in feeds supplemented with citric acid, than the same feeds which had not been supplemented. There was no effect of citric acid supplementation on the mineral or trace element concentrations of whole blood 14 days after the start of supplementation. This reduced the need for additional supplementation and improved efficiency and the environmental characteristics of the feed.

Keywords: Atlantic salmon; citric acid; digestibility; mineral supplementation

5.1 Introduction

Increasing the efficiency of feeds by increasing the absorption of mineral and trace elements (Sugiura et al., 1998) and reducing the mineral content of effluent produced by salmonid aquaculture production are priorities for salmonid producing countries (Cowey & Cho, 1991). The availability of minerals and trace elements is affected by many factors, and the pH of the feed and/or digestive system is one factor that can be changed by modifying the feed. Altering the biochemistry of the feed and the digestive system is possible by supplementing feed with acids to decrease the pH of the digestive system. Research in mammals has shown that the addition of citric acid improves the digestibility of calcium, magnesium, iron, manganese, zinc, potassium and phosphorus (Hohler & Pallauf, 1993; Walter et al., 1998; Ekholm et al., 2003), and affect the transit rate of sodium and potassium through the digestive tract of pigs (Fasshauer & Kienzle, 1995). Supplementing zinc in an organic form, zinc methionine, increased the availability of zinc in feed to channel catfish (*Ictalurus puntatus*, L.) by three time that of zinc sulphate (Paripatananont & Lovell, 1995). In rainbow trout (*Oncorhynchus mykiss*, L.) formic acid increased the availability of iron, phosphorus, calcium and magnesium (Vielma & Lall, 1997), and citric acid increased the availability of phosphorus, calcium, strontium, zinc, copper, iron and magnesium (Sugiura et al., 1998). However no replication was employed in the preliminary observations of Sugiura et al. (1998) and the feeds used contained no mineral supplements. The inclusion of citric acid supplements in feed changed blood mineral concentrations in rainbow trout in a 2-5 weeks (Sugiura et al., 1998), and the authors suggested that further investigation was warranted to identify the effects of citric acid as a dietary supplement to fish meal based feeds. Rainbow tout and

Atlantic salmon have displayed significant differences in the digestibility of phosphorus between feed ingredients (Lall, 1991), so there may be species-specific differences in the effect of citric acid supplementation, and an investigation of the effect of citric acid on Atlantic salmon is required.

The feeds used in the previous experiment (Chapter 4), including 0%, 20%, 40% and 80% of estimated requirement levels of minerals and trace elements, and the resulting ADC and blood values obtained at the end of that experiment provided an opportunity to investigate the effects of supplementing those experimental feeds with citric acid. Citric acid was used as a dietary supplement specifically to increase the acidity of the feed. The effect of citric acid supplementation on the ADC of minerals and trace elements resulting from those different levels of mineral supplementation was observed. Therefore, this experiment was conducted several days after the conclusion of the previous experiment. The aims of this experiment were to observe the effect of supplementing citric acid at 4% and 8% of total feed on the ADC of minerals and trace elements in feeds composed primarily of fish meal and wheat flour and containing 0% to 80% of the estimated mineral and trace element supplementation levels requirements for Atlantic salmon (*Salmo salar*, L.). Citric acid significantly effected ADC and blood mineral content after 7 and 14 days of feeding, respectively, in preliminary observations made on rainbow trout (Sugiura et al., 1998). Therefore, the effect of citric acid inclusion on the concentrations of minerals and trace element in the blood was based on samples taken after a short period of feeding (14 days). The concentrations of minerals and trace element in blood in rainbow trout change significantly over time depending on mineral intake (Rodehutscord et al., 2000), as do ADC in Atlantic salmon(Chapter 4). Therefore, sampling occurred shortly after

providing the experimental feeds to limit any effect of homeostatic mechanisms, such as changes in enzymatic and hormonal pathways associated with digestion and the mobilisation of bodily reserves of minerals and trace elements on ADC.

5.2 Materials and methods

All materials and methods used in this experiment were identical to those in Chapter 4 except for the following.

5.2.1 Fish and experimental systems

This experiment used five hundred Atlantic salmon parr from the previous experiment (Chapter 4), originally obtained from Springfield Hatchery (Springfield, Tasmania, Australia). The fish were maintained in the same tanks and were provided the same experimental feeds they had been assigned in Chapter 4 prior to the start of this experiment. At the start of the experiment the fish were anaesthetised (50 mg l^{-1}, benzocaine), weighed to the nearest 0.1 g, and fork length measured, to the nearest mm. The fish were returned to the same experimental tanks used in the previous experiment, 20 to each tank ensuring there were no significant differences in body weight (117.8 ± 8.6 g). Fish were fasted for two days prior to feeding with the citric acid supplemented diet. Each tank was continued on its respective diet of mineral supplementation as assigned from the previous experiment (Chapter 4). Feeding was done at a common rate for all tanks, 50.0 g per day, to ensure that the same amount of

base feed was provided to each tank. Half the total feed was provided at 9:00 and the remainder at 17:00, at such a rate to ensure no feed was uneaten.

5.2.2 Feeds

Eight experimental feeds (Table 5.1) were formulated by adding citric acid (Anhydrous, Sigma-Aldrich) at either 4% or 8% to four feeds containing 0%, 20%, 40% and 80% concentrations of mineral supplementation, which were used in the previous experiment (Table 5.2). Pelleting proceeded from the feed with the lowest concentration of mineral inclusion and citric acid supplement to those with higher mineral and citric acid supplementation. The first feed pelleted had 0% mineral supplementation and 4% citric acid supplementation, and the next 0% and 8%, then 20% and 4%, 20% and 8%, and so on. The pelleting mill was cleaned of residue between each batch of feed processed.

5.2.3 Sample collection

Faecal samples were taken on day 14, as described in Chapter 4. Blood samples were taken on day 1 and day 14, from three fish in each tank. All samples were frozen and stored at –4 °C. Individual syringes were used for each tank and these were rinsed with an anticoagulant (sodium heparin) prior to drawing blood samples from each of the three fish. All samples were processed at the same time and under the some conditions to prevent any effect of processing.

Table 5.1 Formulation and chemical composition of the experimental feeds

	Feed (Mineral supplement/citric acid supplement)							
	0/4	0/8	20/4	20/8	40/4	40/8	80/4	80/8
Ingredient (g kg^{-1})								
Fish meal	718.7	684.8	718.7	684.8	718.7	684.8	718.7	684.8
Wheat flour	100.3	95.5	100.3	95.5	100.3	95.5	100.3	95.5
Fish oil	67.1	63.9	67.1	63.9	67.1	63.9	67.1	63.9
α-cellulose	60.1	42.9	30.0	0.0	60.1	42.9	30.0	0.0
CMC binder	9.6	9.1	9.6	9.1	9.6	9.1	9.6	9.1
Yttrium oxide marker	1.0	0.9	1.0	0.9	1.0	0.9	1.0	0.9
Mineral and vitamin mix[1]	1.6	15.8	31.6	58.8	1.6	15.8	31.6	58.8
Citric acid	41.7	87.0	41.7	87.0	41.7	87.0	41.7	87.0
Chemical composition								
Dry Matter (g kg^{-1})	844	809	852	816	851	815	811	777
Gross energy (MJ kg^{-1} DM)	18.5	17.8	19.0	18.2	18.9	18.1	18.1	17.4
Crude protein (g kg^{-1} DM)	457	438	487	466	482	462	476	456

[1]Mineral and vitamin mix formulation described in Table 5.2.

Table 5.2 Mineral supplementation (mg kg^{-1}) of experimental feeds

Mineral supplement	Feed (Mineral supplement/citric acid supplement)							
	0/4	0/8	20/4	20/8	40/4	40/8	80/4	80/8
Potassium phosphate dibasic	0.0	0.0	11519	11039	23039	22079	46078	44158
Calcium carbonate	0.0	0.0	1343	1287	2687	2575	5375	5151
Sodium chloride	0.0	0.0	1919	1839	3839	3679	7679	7359
Magnesium carbonate	0.0	0.0	134.4	128.7	268.7	257.5	537.5	515.1
Ferrous sulphate ($FeSO_4$-$7H_2O$)	0.0	0.0	38.4	36.8	76.8	73.6	153.6	147.1
Zinc sulphate ($ZnSO_4$-$7H_2O$)	0.0	0.0	14.40	13.80	28.80	27.60	57.60	55.20
Manganous sulphate ($MnSO_4$-$4H_2O$)	0.0	0.0	15.36	14.72	30.72	29.44	61.44	58.88
Cupric sulphate ($CuSO_4$-$5H_2O$)	0.0	0.0	4.53	4.34	9.05	8.67	18.10	17.35
Cobalt sulphate ($CoSO_4$-$7H_2O$)	0.0	0.0	1.83	1.75	3.66	3.51	7.32	7.01
Potassium iodide	0.0	0.0	0.28	0.26	0.55	0.53	1.11	1.06
Sodium selenate (Na_2SeO_3)	0.0	0.0	0.13	0.12	0.25	0.24	0.50	0.48

All mineral and trace element supplements were sourced from Sigma-Aldrich, Castle Hill, NSW.

5.2.4 Statistical analysis

The statistical methods described in Underwood (1981) and Zar (1984) were applied using SPSS v. 10.0 software (SPSS, 2000). A multivariate analysis of variance was conducted to assess the interaction between sampling day, mineral supplementation, and citric acid supplementation. A two-way analysis of variance was used to identify any interactions between mineral supplementation and citric acid supplementation. A one-way analysis of variance was used to assess the effect of sampling day on the mean mineral and trace element concentrations in blood. Tukey's honestly significant difference test (Tukey's HSD) was used for multiple comparison of means for all data. Significance for all statistical tests was accepted at probability levels of 0.05 or less. One faecal sample contained 10 times more iron than other samples and was considered to be contaminated and was not included in statistical analysis for any element.

5.3 Results

Feed intake was maintained at 50.0 g per day (2.5% BW) for all tanks throughout the experiment, and no tanks exhibited any changes in feed intake or feeding behaviour. No mortalities occurred, and there were no physical signs of disease present in any fish at the end of the experiment.

5.3.1 Effect of supplementation on blood samples

A multivariate analysis of variance indicated no significant interaction ($P > 0.05$) between sampling days, mineral supplementation or citric acid supplementation on the mean concentration of minerals or trace element in blood samples. A two-way analysis of variance indicated no significant differences ($P > 0.05$) in blood mineral or trace element concentrations on samples taken on day 14. There were significant differences between concentrations of mineral and trace elements in blood samples taken on days 1 and 14, with most elements showing a decrease in concentrations over time, except silicon and zinc which rose by 17% and 14%, respectively (Table 5.3).

5.3.2 ADC

There were significant interactions between the mineral and citric acid supplements for ADC of calcium, magnesium, sulphur, phosphorus, aluminium, chromium, molybdenum and nickel (Table 5.4). Aluminium ADC were negative and the interaction between mineral supplementation and citric acid supplementation for this element and chromium were unclear (Figure 5.1). The interaction between molybdenum ADC increased followed increasing mineral supplementation, as did nickel, and each 4% citric acid feed without mineral supplementation had much greater ADC than the 8% citric acid supplemented feeds (Figure 5.2), similar to that displayed by chromium ADC. The interaction between the amount of citric acid supplementation and mineral supplementation were clearer for calcium, phosphorus and magnesium ADC; the feeds with the greater supplementation of citric acid had

Table 5.3 The effect of sampling day on the mean (±SEM, n = 16) concentrations ($mg\ l^{-1}$) of minerals and trace elements in blood samples taken from Atlantic salmon

Mineral	Sample day		F value	P
	1	14	(df=1)	
Al	2.90 (0.1)	4.53 (0.6)	4.07	ns
Ca	66.60 (2.0)	63.99 (2.0)	1.69	ns
Fe	338.32 (66.0)	257.52 (89.7)	41.94	<0.001
K	1893.13 (1,263.9)	1323.30 (1,429.1)	120.57	<0.001
Mg	78.62 (2.0)	62.13 (2.7)	57.53	<0.001
Na	2781.04 (1,512.9)	2371.77 (1,660.5)	52.78	<0.001
P	1470.97 (896.4)	1233.17 (1,095.8)	28.39	<0.001
S	1791.10 (1,291.4)	1515.78 (2,353.3)	20.80	<0.001
Si	4.98 (0.1)	5.84 (0.0)	7.54	0.010
Zn	7.49 (0.1)	8.56 (0.1)	8.75	0.006

Table 5.4 Two-way analysis of variance of mineral supplement level and citric acid supplementation on ADC (%) for Atlantic salmon after 14 days of feeding

Element	Mineral supplement level		Citric acid supplementation		Mineral supplement level * Citric acid supplementation	
	F-value ($df = 1$)	*P*	F-value ($df = 1$)	*P*	F-value ($df = 1$)	*P*
Minerals						
Ca	5.16	0.016	0.23	ns	43.95	<0.001
Fe	0.97	ns	2.40	ns	0.86	ns
K	0.38	0.001	34.00	<0.001	1.60	ns
Mg	5.97	0.010	0.03	ns	31.96	<0.001
Na	0.07	ns	2.83	ns	1.40	ns
P	1.63	ns	0.20	ns	16.22	0.001
S	3.14	0.002	37.51	<0.001	7.65	0.010
Trace elements						
Al	1.07	ns	2.32	ns	14.82	0.001
B	1.52	ns	1.00	ns	0.82	ns
Cr	3.74	0.041	0.46	ns	18.73	0.001
Cu	7.70	0.004	0.01	ns	0.39	ns
Mn	6.44	0.008	4.49	ns	3.14	ns
Mo	6.60	0.007	1.17	ns	29.20	<0.001
Ni	0.58	ns	0.99	ns	108.51	<0.001
Si	2.58	ns	0.12	ns	1.36	ns
Zn	13.06	<0.001	0.01	ns	1.52	ns

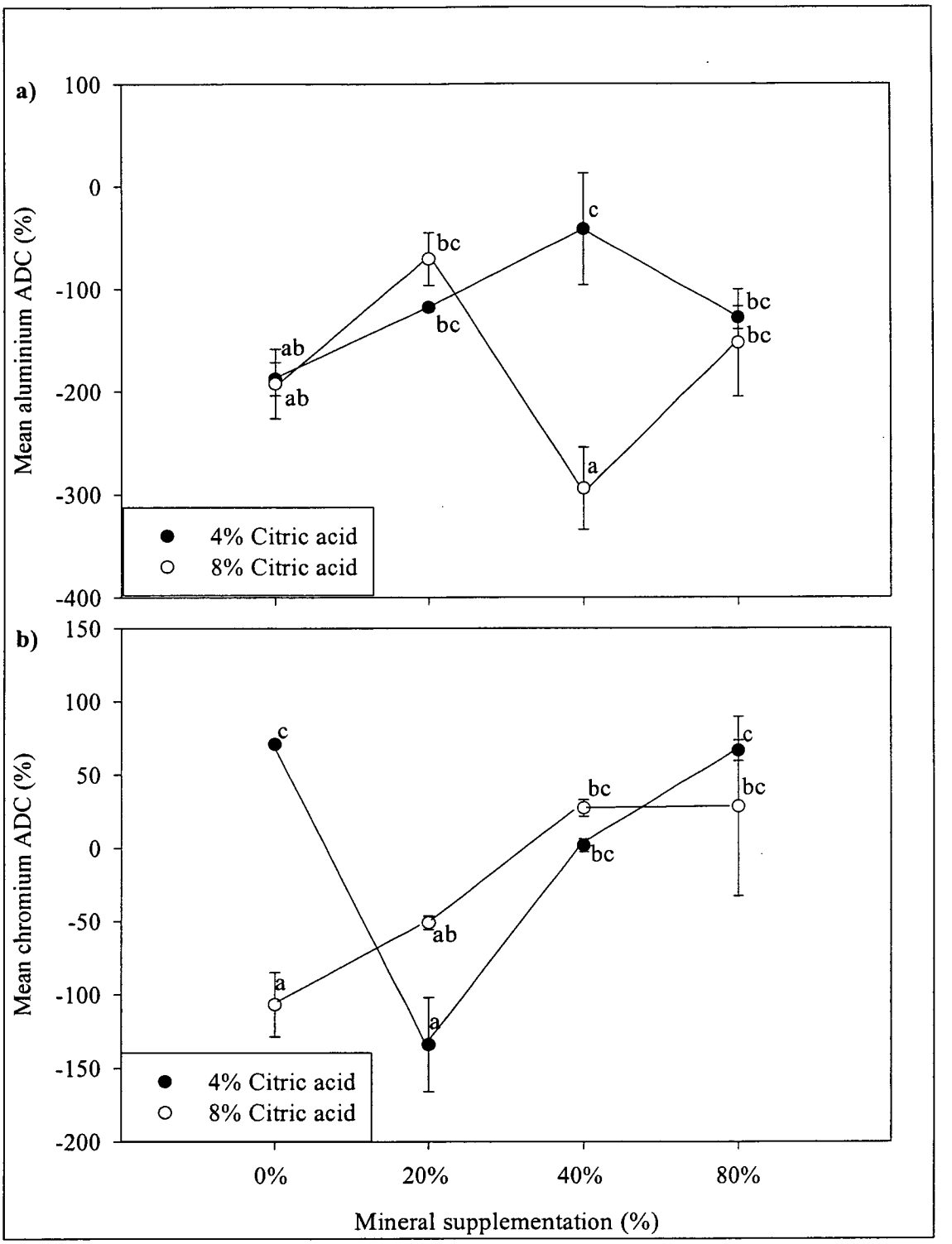

Figure 5.1 The effects of mineral supplementation and citric acid supplementation on mean (±SEM, $n = 2$) a) aluminium and b) chromium ADC (%) in Atlantic salmon, after 14 days feeding. Means with the same superscript were not significantly different (Tukey's HSD).

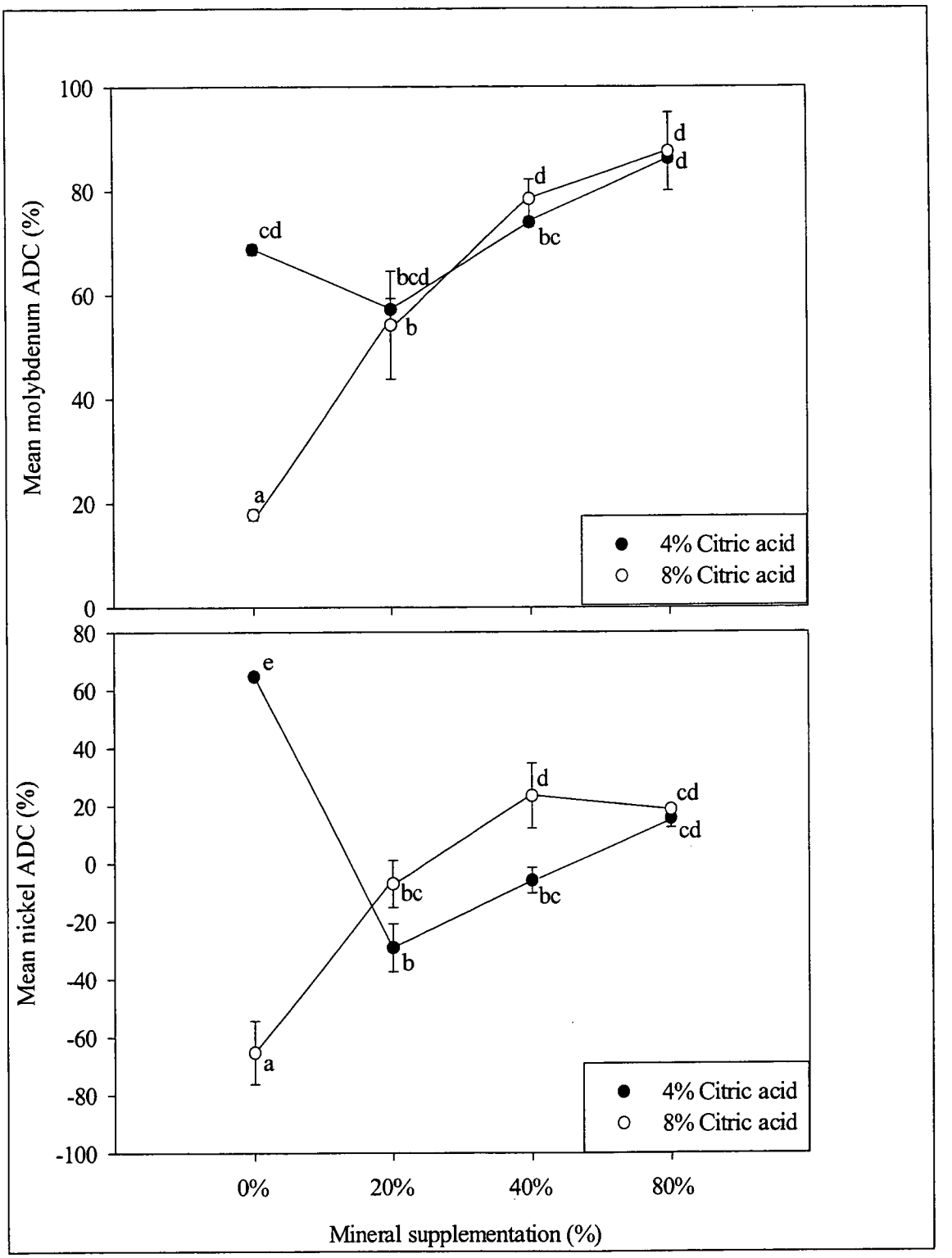

Figure 5.2 The effects of mineral supplementation and citric acid supplementation on mean (±SEM, $n = 2$) a) molybdenum and b) nickel ADC (%) in Atlantic salmon, after 14 days feeding. Means with the same superscript were not significantly different (Tukey's HSD).

lower ADC for each of these minerals as mineral supplementation increased (Figure 5.3). The interaction between citric acid supplementation and mineral supplementation in sulphur ADC changed with the addition of supplemental minerals, the addition of 20% of mineral resulted in a much lower ADC for sulphur in the feed with 8% citric acid; potassium ADC varied with mineral supplement and citric acid supplementation, but there was no significant interaction ($P > 0.05$) between these factors (Figures 5.4). Zinc and manganese ADC decreased with increases in feed supplement level (Figure 5.5). There were no significant differences in the ratio of marker in the feed to that in the faeces, which suggested that there were no interactions between the marker and the minerals and trace elements.

As the fish and the experimental feed without mineral supplementation had been used to calculate ADC under nearly identical conditions, with the inclusion of citric acid the only change, the ADC calculated from the experimental feeds without mineral supplementation and 4% and 8% citric acid supplementation were compared with those calculated from the final faecal collection taken at the end of the previous experiment (Chapter 4). The number of significant interactions between mineral supplementation and citric acid supplementation prevented a comparison of those experimental feeds which contained mineral supplements. The ADC for calcium, phosphorus, and zinc were significantly higher in those feeds with 4 and 8% citric acid, significantly lower for iron, potassium, sodium, molybdenum, and nickel and there were no significant differences ($P > 0.05$) for magnesium, sulphur, aluminium, boron, copper, manganese, or silicon (Table 5.5).

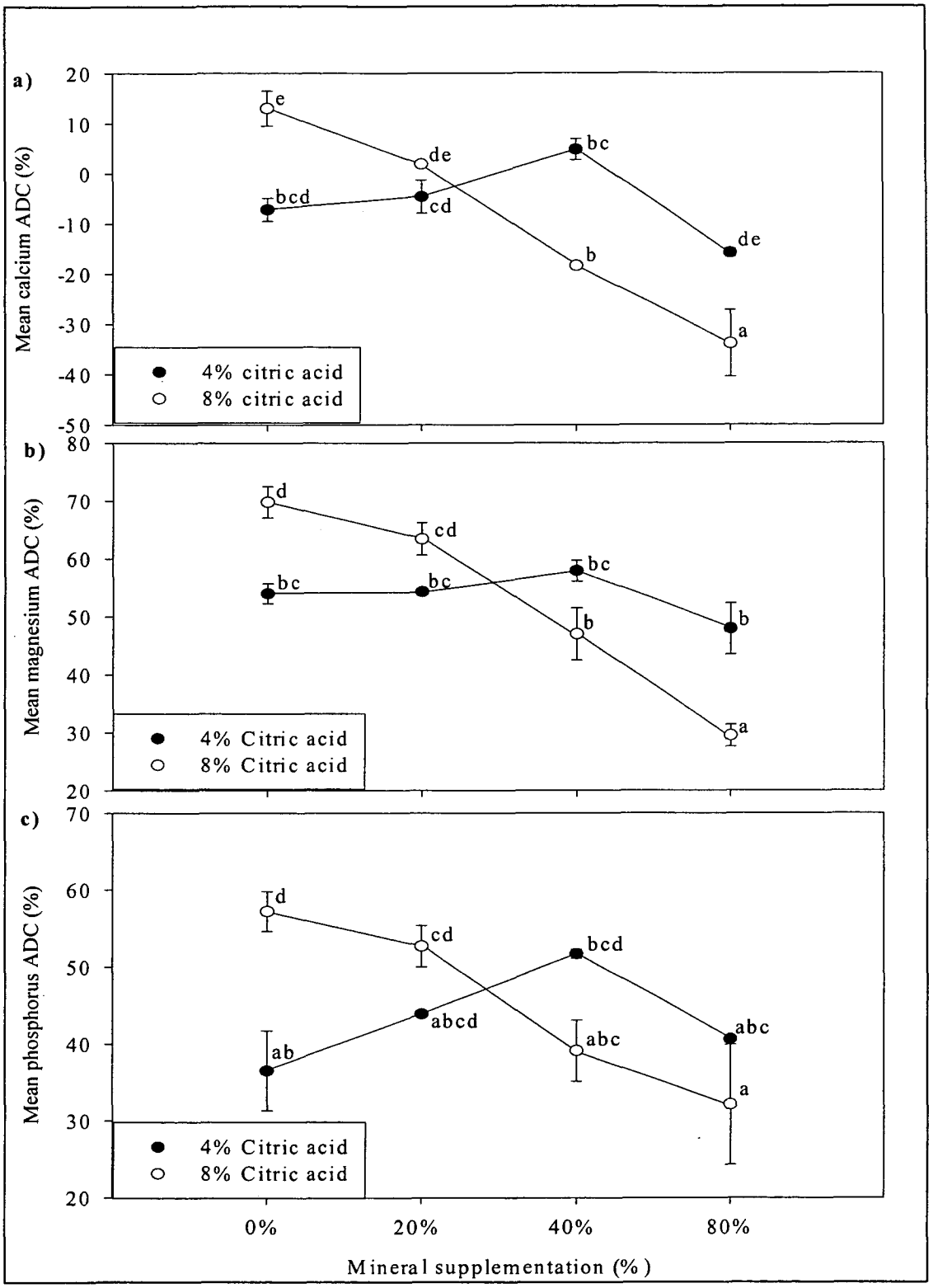

Figure 5.3 The effects of mineral supplementation and citric acid supplementation on mean (±SEM, $n = 2$) a) calcium, b) magnesium and c) phosphorus ADC (%) in Atlantic salmon, after 14 days feeding. Means with the same superscript were not significantly different (Tukey's HSD).

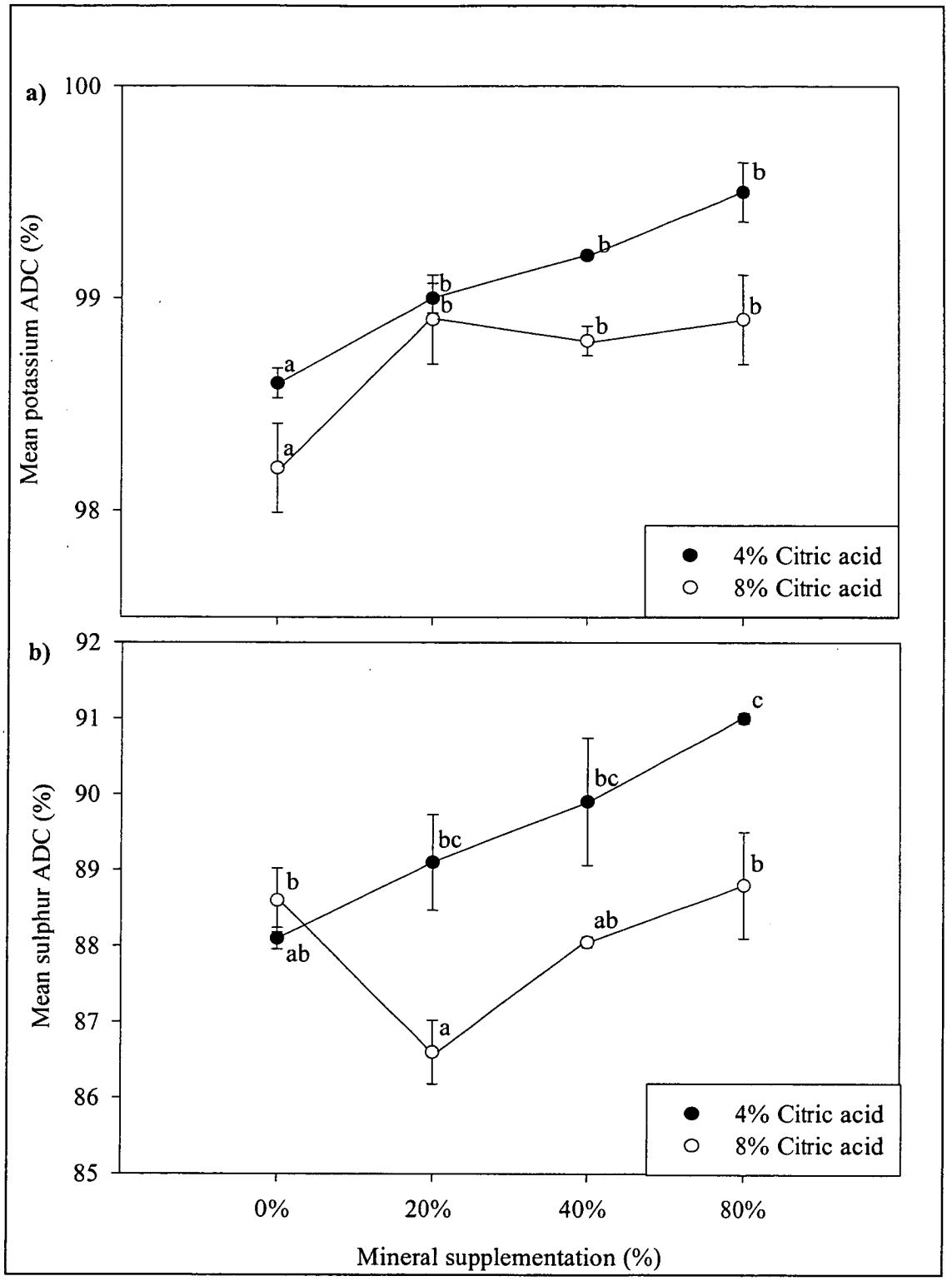

Figure 5.4 The effects of mineral supplementation and citric acid supplementation on mean (±SEM, $n = 2$) a) potassium and b) sulphur ADC (%) in Atlantic salmon, after 14 days feeding. Means with the same superscript were not significantly different (Tukey's HSD).

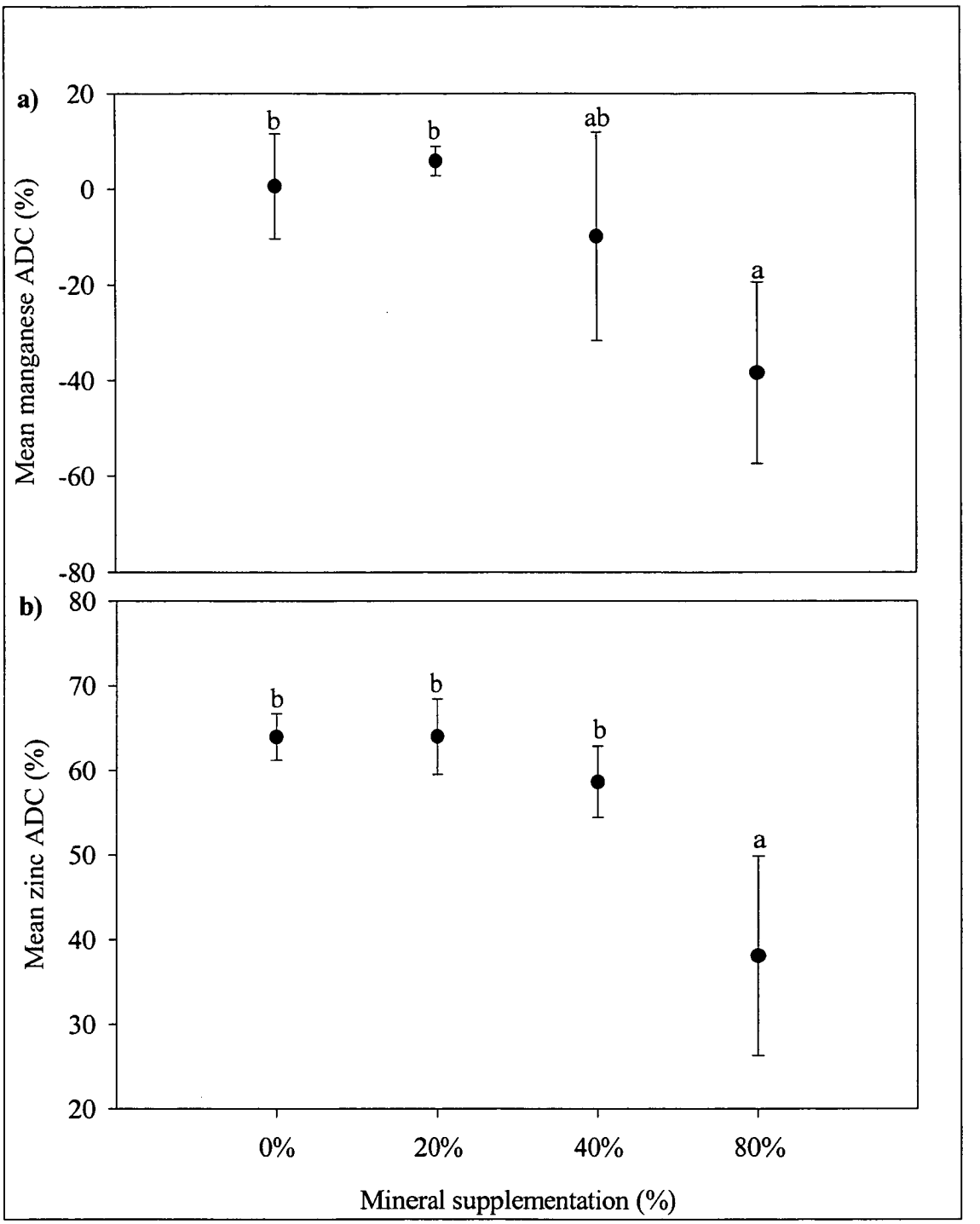

Figure 5.5 Effect of mineral supplementation, regardless of citric acid supplementation, on mean (±SEM, $n = 4$) a) manganese and b) zinc ADC (%) in Atlantic salmon, after 14 days feeding. Means with the same superscript were not significantly different (Tukey's HSD).

Table 5.5 A comparison of mean (±SEM, n = 8) mineral and trace element ADC (%) derived from fish meal based experimental feeds with 0%, 4% and 8% citric acid supplementation and no mineral supplementation

Element	Citric acid supplementation			F-value	P
	0%	4%	8%	(df = 1)	
Minerals					
Ca	-18.66[a] (12.22)	-7.22[ab] (2.31)	13.04[b] (3.51)	7.050	0.027
Fe	-59.95[b] (53.44)	-26.95[b] (1.79)	-275.70[a] (6.30)	21.042	0.002
K	99.33[b] (0.25)	98.66[a] (0.04)	98.26[a] (0.19)	18.628	0.003
Mg	62.67 (7.86)	54.03 (1.75)	69.77 (2.74)	2.902	ns
Na	92.54[b] (1.30)	77.23[a] (0.68)	73.97[a] (2.04)	170.971	<0.001
P	23.26[a] (10.85)	36.55[ab] (5.20)	57.16[b] (2.60)	9.879	0.013
S	96.55 (7.71)	88.10 (0.10)	88.58 (0.42)	1.890	ns
Trace elements					
Al	-517.17 (238.11)	-187.70 (16.25)	-192.21 (33.79)	3.128	ns
B	-225.59 (192.79)	73.27 (0.66)	49.65 (34.94)	3.676	ns
Cu	23.04 (28.42)	61.67 (0.67)	51.17 (2.46)	2.397	ns
Mn	-27.18 (48.31)	9.90 (3.57)	-8.71 (1.48)	.661	ns
Mo	74.06[b] (10.27)	68.68[b] (1.02)	17.76[a] (1.07)	33.286	0.001
Ni	81.89[b] (7.84)	64.66[b] (0.54)	-65.32[a] (10.86)	262.814	<0.001
Si	94.95 (2.76)	98.85 (0.18)	98.89 (0.07)	3.352	ns
Zn	28.16[a] (15.54)	62.29[ab] (3.04)	65.60[b] (1.64)	8.761	0.017

The mean ADC for feed without citric acid supplementation were calculated from faecal samples taken at the end of the previous experiment (see Chapter 4).

5.4 Discussion

The methods employed in the present experiment proved useful for determining the effect of citric acid supplementation on mineral and trace element ADC values and concentrations in blood samples. The decision to make the assessment of the effect of citric acid is based on the significant changes observed in ADC and blood mineral concentrations over time in Atlantic salmon (Chapter 4). Short term experiments such as this one can provide a snapshot of the effects of feed supplements such as citric acid. Taking samples at day 14 should have limited the effect of homeostatic mechanisms on the concentration of minerals and trace elements in the blood samples and on ADC (Chapter 4). However, additional research into the usefulness of other tissues or methods of measuring the effect of supplementation is warranted, to identify those methods that increase the effect size of any dietary supplementation treatments and overcome the effects of homeostatic mechanisms. Vielma et al. (1999) suggested that whole body measurements alone may not be useful for determining the effect of citric acid supplementation on mineral digestibility, and the previous experiment proved that tissue samples are useful for determining mineral availabilities. Sections of the gastrointestinal tract provided a means of assessing the effect of macronutrient composition in feed on apparent nutrient absorption in Atlantic salmon (Krogdahl et al., 1999), and may prove useful in determining the effect of dietary supplementation on mineral absorption too.

5.4.1 Blood mineral concentrations

The analysis of whole blood to assess changes in the bioavailability of minerals resulting from citric acid supplementation provided no evidence of significant differences between the inclusion of 4% and 8% citric acid. The fall in mineral and trace element concentrations from the blood samples taken indicated either increasing demands for these nutrients in the growing fish and/or decreasing availability (Shearer et al., 1994; Andersen et al., 1997). The increase in zinc concentration in the blood could have resulted from increased supplementation in the feed, as there was a significant effect of mineral supplementation on the zinc ADC calculated (Apines et al., 2001). Changes in the plasma fraction of the whole blood may have been masked by the blood cell fraction. However, practical necessities prevented taking blood samples large enough to provide plasma for analysis. Sugiura et al. (1998) observed some differences in the concentrations of calcium and phosphorus in blood plasma in rainbow trout fed feeds supplemented with 2% and 5% of citric acid over a five-week period, and the 5% inclusion displayed somewhat high concentrations of these elements in the blood plasma. However, these results were from a preliminary work with no replicates, and there were no differences in calcium between the control feed with 0% citric acid and the feed containing 2% citric acid and the 2% citric acid feed had lower phosphorus and potassium in the blood plasma, as were found in the present experiment.

5.4.2 ADC

A number of factors can account for the interactions between mineral supplementation and citric acid inclusion on ADC calculated in supplemented minerals and those not specifically supplemented, such as nickel, molybdenum, aluminium and chromium. The ADC calculated for these elements tended to increase with supplement level and must indicate synergistic effects of other supplemented minerals on the digestibility of these trace elements. The ADC calculated for potassium and sulphur, which rise with increasing supplementation, tended to decrease with the doubling of citric acid supplementation and may indicate an effect of changing gut pH on the solubility of these elements in phosphate and sulphate compounds present in the feed. For those feeds with no mineral supplementation doubling the citric acid supplementation significantly reduced the ADC of molybdenum and chromium, and increased the digestibility of calcium, phosphorus and magnesium. Acids may improve mineral and trace element digestibility by solubilising supplemental mineral compounds and bone-mineral in the fish meal portion of the feed, as suggested by Sugiura et al. (1998), or by acting as chelators, as suggested by Maenz et al. (1999), thus improving the availability of minerals supplemental compounds and the plant-based portion of these feeds. Providing organic forms of supplements, as opposed to inorganic forms, may increase the availability and digestibility of many trace elements, as has been shown in channel catfish with the use of zinc methionine (Paripatananont & Lovell, 1995).

Comparing the ADC calculated from feeds without mineral supplementation in the previous experiment, run under identical conditions with the same feeds (without citric acid), and the results obtained in the present experiment revealed several

significant differences (Table 5.5). The ADC were generally lower than those Sugiura et al. (1998) calculated for rainbow trout fed semi-purified feed with up to 5% citric acid. The feeds in the present study all contained minerals and trace elements in concentrations near or above estimated requirements, and decreases in ADC may reflect regulatory processes resulting in the excretion of those elements at the gastrointestinal level present above requirements. The feeds contained iron in excess of requirements, 226 – 350% of the highest requirement established for Atlantic salmon (Waagbø et al., 1996), and the negative ADC for this mineral indicated excretion of the excess. Zinc concentrations in the feed were 68 – 86% of the highest requirement established for Atlantic salmon (67 mg kg^{-1}) (Maage & Julshamn, 1993), and significant differences in ADC were calculated for zinc at the highest inclusion level. The differences may be species specific, as differences have been proven to exist between the apparent phosphorus availability values for rainbow trout, Atlantic salmon and channel catfish for a given feed ingredient, and apparent phosphorus availability in trout was maximal when experimental feeds contained phosphorus concentrations near the published requirements (Riche & Brown, 1996). Therefore, it is likely that there are species differences for the digestibility of other elements.

The reasons for supplementing feeds with acids were to increase the digestibility of minerals and trace elements, improve feed efficiency, and limit the loss of these minerals in effluent water. For elements such as phosphorus, required in high amounts by salmon (Ketola, 1975; Rodehutscord & Pfeffer, 1995) and a major pollutant (Cho & Bureau, 2001; Coloso et al., 2003) these small increases in digestibility can prove to be economically and environmentally beneficial. For other minerals and trace elements present in quantities above estimated requirements and

not of environmental concern the citric acid supplementation would provide little benefit commercially, or in feed efficiency.

5.5 Conclusions

Citric acid supplementation improved the ADC of potassium and sulphur, and when combined with mineral supplements improved the ADC of aluminium, chromium, molybdenum, nickel, calcium, magnesium and phosphorus. There was no apparent effect of citric acid supplementation on the mineral or trace element concentrations of whole blood on samples taken 14 days after the start of supplementation. Whole blood samples did not provide a useful measure of the effect of citric acid supplementation on mineral bioavailability in the short term. The effects of citric acid supplementation can be effectively measured in ADC calculated after short term exposure to citric acid supplementation. These results confirm the effect of citric acid on mineral and trace element digestibility in the short term, and support the need for longer term investigations using this dietary supplement.

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Chapter 6

The apparent digestibility of crude protein, energy and micro-nutrients from white lupin (*Lupinus albus*), Australian sweet lupin (*Lupinus anagustifolius* [Gungurru cultivar]) and soybean (*Glycine max*) protein concentrate in juvenile Atlantic salmon (*Salmo salar* L.)

Abstract

The apparent digestibility coefficients (ADC) of energy, crude protein, minerals and trace elements for *Lupinus albus* meal, *Lupinus anagustifolius* meal, and a soybean (*Glycine max*) protein concentrate were measured. There were no significant differences in ADC for energy or protein between the ingredients, each contained approximately 17.0 MJ kg^{-1} DM of digestible energy and 403 g kg^{-1} DM of digestible crude protein. There were significant differences in ADC values for calcium, potassium, magnesium, sodium, phosphorus, sulphur, aluminium, boron, cobalt, copper, manganese, molybdenum, nickel, silicon, vanadium, and zinc between the three ingredients. *Lupinus albus* contained 15 times more manganese than the *Lupinus anagustifolius*, and contained significantly more potassium, sodium, molybdenum, nickel and zinc. This was attributed to different growing conditions experienced by the plants. A number of ADC values fell outside the 1-100% range, resulting from the experimental ingredients being significantly different from the reference feed in the concentration of a number of minerals and trace elements. These ADC values outside the normal range indicated the antagonistic or synergistic effects of the experimental ingredients on the ADC of the reference feed. Alternative methods may be needed to assess mineral and trace element ADC in ingredients with such dissimilar nutrient concentrations. Mineral retention is one such method, and provided an indication of the bioavailability of the minerals and trace elements in each of these ingredients.

Keywords: Aquaculture feeds; alternative protein sources, Atlantic salmon; fish meal replacement; minerals and trace element digestibility

6.1 Introduction

Increasing demand for high protein feeds for salmonids has lead to research in alternative protein sources derived from plants such as soybeans (Ketola, 1975; Pongmaneerat & Watanabe, 1992; Watanabe & Pongmaneerat, 1993; Kaushik et al., 1995; Olli et al., 1995; Refstie et al., 1998; Storebakken et al., 1998a; Carter & Hauler, 2000) lupins (Carter & Hauler, 2000; Farhangi & Carter, 2001a, b; Glencross et al., 2002), and other grain and legume products (Hardy & Sullivan, 1983; Carter & Hauler, 2000). Mineral content for a number of feed ingredients, including soybeans, has been published (NCR, 1993), but little information exists on the apparent digestibility coefficients (ADC) of minerals from these alternative plant protein sources. Soybean meal and concentrates are known to contain less calcium and phosphorus than fish meal, and supplementing trout diets that contain soybean has proven effective in preventing deficiencies for these two minerals (Ketola, 1975) and similarly for zinc (Gomes & Kanshik, 1993). Therefore, it is necessary to measure the amount of digestible mineral and trace elements provided by high-protein plant ingredients, to better design future experiments that make use of these common high-protein substitutes for fish meal.

The information available on mineral and trace element ADC for alternative protein sources have been derived for coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) using a purified basal feed (Sugiura et al., 1998a). Purified feeds were chosen to minimize unknown variables, such as composition, property and quality factors. However, it is also necessary to measure the effects of these protein substitutes on mineral and trace element ADC when combined with common feed

ingredients. Plant ingredients used to replace fish meal as a protein source often contain anti-nutritional factors such as phytate which limit the digestibility of phosphorus, calcium, magnesium and zinc in salmon (Storebakken et al., 1998b; Vielma et al., 2000). Therefore, in the present experiment purified ingredients were not used, and a reference salmon feed, composed primarily of fish meal and wheat flour (from the previous digestibility experiments) was used.

The aim of this experiment was to assess the suitability of the ingredients, *Lupinus albus*, *Lupinus anagustifolius*, [Gungurru] and *Glycine max* for further research (e.g., Chapter 7), specifically to identify the digestible energy, digestible crude protein, mineral and trace element ADC of two readily available varieties of lupin meal and a soybean meal for Atlantic salmon. This was a preparatory experiment, and as previous investigations showed very low variability in ADC determination between tanks (Chapters 2, 4, and 5) for most minerals and trace elements, tank replication was limited to one tank and achieved by taking multiple samples over time. In addition, relatively large numbers of fish were acclimated for over two weeks to the experimental system to limit any effects of stress, resulting from transfer and the establishment of feeding hierarchies, on feeding and ADC calculations.

6.2 Materials and methods

6.2.1 Experimental ingredients

Two lupins, *Lupinus albus*, and *Lupinus anagustifolius* [Gungurru cultivar], and a commercial soybean (*Glycine max*) protein concentrate, Soycomil® P, were used as

high-protein fish meal replacements. The lupin meals were milled from pre-cleaned, de-hulled lupin seed so that 100% of the meal passed through a 2.55 mm screen. The two species of lupin and the soybean protein concentrate differed significantly in energy, crude protein and crude lipid content (Table 6.1). The soybean protein concentrate had greater concentrations of calcium, potassium, magnesium, sulphur, nickel and aluminium, while *Lupinus anagustifolius* meal contained significantly less calcium and phosphorus than the other two ingredients (Table 6.1). The three ingredients did not differ significantly ($P > 0.05$) in aluminium, arsenic or selenium content. *Lupinus albus* had the lowest concentration of magnesium, and 12 and 6 times more manganese than the soybean protein concentrate and *Lupinus anagustifolius* ingredients, respectively (Table 6.2).

6.2.2 Experimental feeds

Four experimental feeds were formulated by replacing 30% of a reference salmon feed (REF) with one of the three test ingredients (Table 6.3) (Cho et al., 1982). The REF feed contained greater amounts of gross energy and crude protein than the experimental feeds, ANA and SOY contained lower gross energy than the other two feeds, and the REF and SOY feeds had greater concentrations of crude protein than the lupin feeds. The reference feed was supplemented with minerals and trace elements to meet the estimated requirements of salmonids (Tables 6.4) as did each experimental feed (Tables 6.5 and 6.6). The plant meals were thoroughly mixed into the moist reference feed, and each feed was pelleted at room temperature with a 3 mm

Table 6.1 Mean (±SEM, $n = 3$) macronutrient and mineral content (mg kg^{-1}) of the experimental ingredients

Parameter	Ingredient			F value	*P*
	L. albus	*L. anagustifolius*	*G. max*	(*df*=3)	
Macronutrients					
Gross energy (MJ kg^{-1} DM)	19.33[c] (0.14)	18.89[b] (0.13)	17.82[a] (0.09)	145.13	<0.001
Crude protein (g kg^{-1} DM)	328[a] (5)	364[b] (4)	385[c] (4)	126.76	<0.001
Crude lipid (g kg^{-1} DM)	66[b] (1)	60[a] (1)	58[a] (1)	52.00	<0.001
Minerals					
Ca	1080.88[a] (80.05)	1569.42[b] (76.64)	5518.39[c] (138.43)	1694.71	<0.001
Fe	38.13[a] (3.77)	42.10[a] (2.17)	122.60[b] (1.56)	958.77	<0.001
K	11214.59[b] (123.92)	10297.33[a] (126.71)	23320.42[c] (240.84)	5317.68	<0.001
Mg	1190.26[a] (14.05)	1946.34[b] (67.80)	3941.00[c] (248.80)	272.50	<0.001
Na	122.36[c] (1.73)	61.89[b] (3.13)	21.45[a] (2.69)	1158.52	<0.001
P	4096.30[a] (95.68)	3882.70[a] (117.92)	9537.81[b] (35.08)	3805.91	<0.001
S	2839.16[a] (36.41)	3025.95[b] (19.19)	5573.20[c] (31.48)	7819.42	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

Table 6.2 Mean (±SEM, $n = 3$) trace element content (mg kg^{-1}) of the experimental ingredients

Element	Ingredient			F value	*P*
	L. albus	*L. anagustifolius*	*G. max*	(*df*=3)	
Al	8.36 (3.60)	3.31 (2.87)	4.85 (8.40)	0.65	ns
As	1.36 (1.09)	0.89 (0.94)	1.50 (0.96)	0.31	ns
B	19.44^{a} (1.09)	19.46^{a} (0.97)	28.92^{b} (0.70)	102.69	<0.001
Co	0.72^{b} (0.11)	0.09^{a} (0.17)	0.03^{a} (0.06)	28.99	0.001
Cu	8.82^{b} (0.26)	5.38^{a} (0.29)	15.10^{c} (0.46)	594.61	<0.001
Mn	3644.26^{c} (39.50)	235.44^{b} (29.32)	52.61^{a} (5.51)	15031.40	<0.001
Mo	29.87^{b} (1.42)	4.42^{a} (1.32)	1.71^{a} (2.96)	173.72	<0.001
Ni	3.82^{c} (0.07)	2.15^{b} (0.06)	0.33^{a} (0.15)	934.89	<0.001
Se	4.39 (1.47)	3.61 (1.04)	3.48 (1.07)	0.49	ns
Si	1.24^{a} (0.40)	0.80^{a} (0.51)	3.41^{b} (1.30)	8.35	0.018
Sr	4.29^{a} (0.04)	8.37^{c} (0.15)	6.18^{b} (0.16)	771.46	<0.001
V	9.07^{a} (0.84)	11.16^{a} (1.68)	21.81^{b} (0.99)	93.53	<0.001
Zn	42.05^{b} (2.49)	35.64^{a} (0.90)	37.79^{a} (0.83)	12.43	0.007

Means with the same superscript were not significantly different (Tukey's HSD).

Table 6.3 Ingredient and chemical composition of the experimental feeds

Parameter	Source[1]	Feed[2]			
		REF	ALB	ANA	SOY
Ingredient composition (mg kg^{-1})					
Fish meal (Peruvian)	Skretting	700.0	490.0	490.0	490.0
Anagustifolius lupin meal	M.C. Croker	0.0	300.0	0.0	0.0
Albus lupin meal	M.C. Croker	0.0	0.0	300.0	0.0
Soycomil® P	Gibson's	0.0	0.0	0.0	300.0
Fish oil (Peruvian)	Skretting	150.0	105.0	105.0	105.0
Wheat flour	Gibson's	59.0	41.3	41.3	41.3
Yttrium oxide (Y_2O_3)	Sigma-Aldrich	1.0	0.7	0.7	0.7
CMC binder	Sigma-Aldrich	10.0	7.0	7.0	7.0
Mineral and vitamin mix	See table 6.2	80.0	56.0	56.0	56.0
Chemical composition (mg kg^{-1} DM)					
Gross energy (MJ kg^{-1}DM)		20.1	20.3	19.3	19.2
Crude protein (g kg^{-1} DM)		454.0	434.2	426.7	475.6

[1]Skretting and Gibson's (Cambridge, Tasmania); Sigma-Aldrich (Castle Hill, NSW) and M.C. Croker (Cootamundra, NSW).

[2]REF: reference feed

ALB: reference feed plus 30% *Lupinus albus* meal

ANA: reference feed plus 30% *Lupinus anagustifolius* [Gungurru cultivar] meal

SOY: reference feed plus 30% *Glycine max* (Soycomil® P) from a soybean protein concentrate

Table 6.4 Mineral and vitamin supplement mix

Mineral and vitamin supplements	mg kg^{-1}
Potassium phosphate dibasic	60000
Calcium carbonate	7000
Sodium chloride	10000
Magnesium carbonate	500
Ferrous sulphate	200
myo-Inositol	300
Zinc sulphate	75
Manganous sulphate	80
DL alpha tocopherol acetate	50
Stay-C® (L-ascorbyl 2 polyphosphate)	50
Cupric sulphate	23.6
Calcium D-pantothenate	21.7
Nicotinic acid	10.0
Cobalt sulphate	9.5
Retinol acetate (2800000 IU/g)	5.0
Riboflavin	4.0
Pyridoxine HCl	3.7
Vitamin D_3 powder (850000 IU/g)	2.8
Menadone sodium bisulphate	2.0
Potassium iodide	1.4
Thiamin HCl	1.1
Folate	1.0
Sodium selenate	0.7
d-Biotin	0.15
Vitamin B_{12}	0.01
Choline chloride	1333

All mineral and trace element supplements were sourced from Sigma-Aldrich (Castle Hill, NSW) except for Stay-C® which was sourced from Roche Vitamins Australia (Frenchs Forest, NSW).

Table 6.5 Mean (±SD, $n = 3$) mineral content (mg kg^{-1}) of the experimental feeds

Element	Feed				F value	*P*
	REF	ALB	ANA	SOY	(*df*=3)	
Ca	26388.54[b] (983.78)	20524.42[a] (844.04)	20280.83[a] (294.08)	21693.01[a] (164.57)	54.16	<0.001
Fe	468.53[b] (62.09)	247.07[a] (21.32)	218.17[a] (3.29)	304.83[a] (104.17)	9.90	0.005
K	25199.76[c] (429.07)	21854.97[ab] (1027.43)	21170.87[a] (457.31)	23281.08[b] (439.02)	23.18	<0.001
Mg	2148.47[b] (5.31)	2017.09[a] (30.81)	2128.11[b] (20.74)	2780.32[c] (47.06)	396.75	<0.001
Na	10920.00[b] (338.75)	8835.19[a] (335.37)	8437.53[a] (83.08)	8660.10[a] (116.72)	63.99	<0.001
P	26397.12[b] (1195.18)	21726.97[a] (423.92)	20493.23[a] (166.98)	21833.20[a] (200.72)	48.21	<0.001
S	6433.40[c] (57.78)	5478.44[a] (77.80)	5486.48[a] (80.69)	5865.17[b] (26.04)	146.23	<0.001

Means with the same superscript are not significantly different (Tukey's HSD).

Table 6.6 Mean (±SD, n = 3) trace element content (mg kg^{-1}) of experimental feeds

Element	Feed				F value	P
	REF	ALB	ANA	SOY	(df=3)	
Al	17.70^{b} (3.94)	9.59^{a} (0.98)	12.81^{ab} (1.71)	25.10^{c} (2.23)	22.47	<0.001
B	25.87^{a} (4.23)	29.04^{ab} (0.67)	35.31^{b} (1.64)	34.41^{b} (3.58)	7.11	.012
Ba	4.85^{b} (0.16)	4.35^{a} (0.07)	5.12^{b} (0.13)	8.67^{c} (0.10)	784.03	<0.001
Cd	0.57^{b} (0.02)	0.40^{a} (0.05)	0.38^{a} (0.02)	0.42^{a} (0.03)	20.92	<0.001
Co	2.34^{b} (0.77)	1.50^{ab} (0.52)	0.92^{a} (0.10)	1.08^{ab} (0.30)	5.05	0.030
Cu	11.06 (1.04)	15.30 (11.62)	11.14 (4.67)	11.41 (3.20)	0.30	ns
Mn	73.72^{a} (14.46)	934.78^{c} (49.22)	163.60^{b} (18.40)	74.01^{a} (14.81)	656.23	<0.001
Mo	1.17 (0.37)	1.75 (0.19)	1.19 (0.17)	1.68 (0.38)	3.36	ns
Ni	2.38^{ab} (0.40)	1.78^{a} (0.08)	1.19^{ab} (0.06)	2.74^{b} (0.91)	5.55	0.023
Si	115.53^{b} (17.16)	62.81^{a} (10.58)	63.85^{a} (4.58)	74.52^{a} (3.44)	16.79	0.001
V	2.53 (0.31)	2.04 (0.35)	2.58 (0.49)	2.21 (0.11)	1.72	ns
Y	834.02^{b} (17.02)	618.38^{a} (51.10)	551.44^{a} (2.84)	573.43^{a} (31.00)	51.99	<0.001
Zn	70.25 (12.49)	55.99 (3.03)	58.38 (6.90)	58.30 (1.08)	2.32	ns

Means with the same superscript are not significantly different (Tukey's HSD).

die, on a California Laboratory Pellet Mill (CL-2 laboratory pellet mill, California Pellet Mill Co., San Francisco, U.S.A.). After pelleting an experimental feed, the mill was cleaned of any residual feed by running oats through the mill, and drilling out the pellet die. The pelleted feeds were oven-dried at 40° C for over 24 h, and stored in a cold room at 2.7° C until required.

6.2.3 The experimental system

The experiment was conducted at the School of Aquaculture, University of Tasmania. The twelve 300-L, cylindrical, tanks comprising the experimental system were housed in an unheated research building, although water temperature was maintained at 15.0° C. Freshwater was supplied to each tank in a partial replacement system with a continuous replacement of approximately 10% per day from the municipal water supply. The system supplied filtered water to each tank at an average flow rate of 6 l min^{-1}. Water quality characteristics (dissolved oxygen, oxygen saturation, chlorine, pH, ammonia, nitrate and nitrite) were monitored in experimental tanks, taken at random on a daily basis, and within the reservoir tank throughout the experiment to ensure water quality remained within the limits recommended for Atlantic salmon (Wedemeyer, 1996). Photoperiod was not controlled.

Atlantic salmon (*Salmo salar* L.) parr were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia) and stocked at 20 fish per tank. These fish were acclimatised to the system for 15 days. At the start of the experiment fish were anaesthetised (50 mg l^{-1}, benzocaine) and weight for each fish was measured to the

nearest 0.1 g. Eighty fish were distributed to four tanks to ensure that there were no significant differences between group mean weights (175.6 ±24.6 g). Fish were switched from a commercial diet to the experimental feeds 14 days prior to the start of faecal sampling, and were fed 2.0% body weight (BW), as determined from the average biomass of each tank, over two periods per day (9:00 and 17:00).

Fish faeces were collected for 18 h on five consecutive nights. Collection started one h after the evening feed and ending prior to the next morning feed, using a modification of the Guelph design (Cho & Slinger, 1979), as described in Carter and Hauler (2000) and previous chapters (Figure 6.1). Faecal samples were frozen immediately after collection and stored at -4° C. After all faecal samples were obtained and frozen, the entire set were freeze-dried at -10 to -12° C, and -100 to -110 kPa pressure, for several days, until all samples reached a constant weight. The samples collected on each day were kept separate for analysis ($n = 5$).

6.2.4 Analytical procedures

Standard methods were used to determine dry matter (oven dried at 100 °C to a constant weight), gross energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid) and nitrogen (Kjeldahl using selenium catalyst).

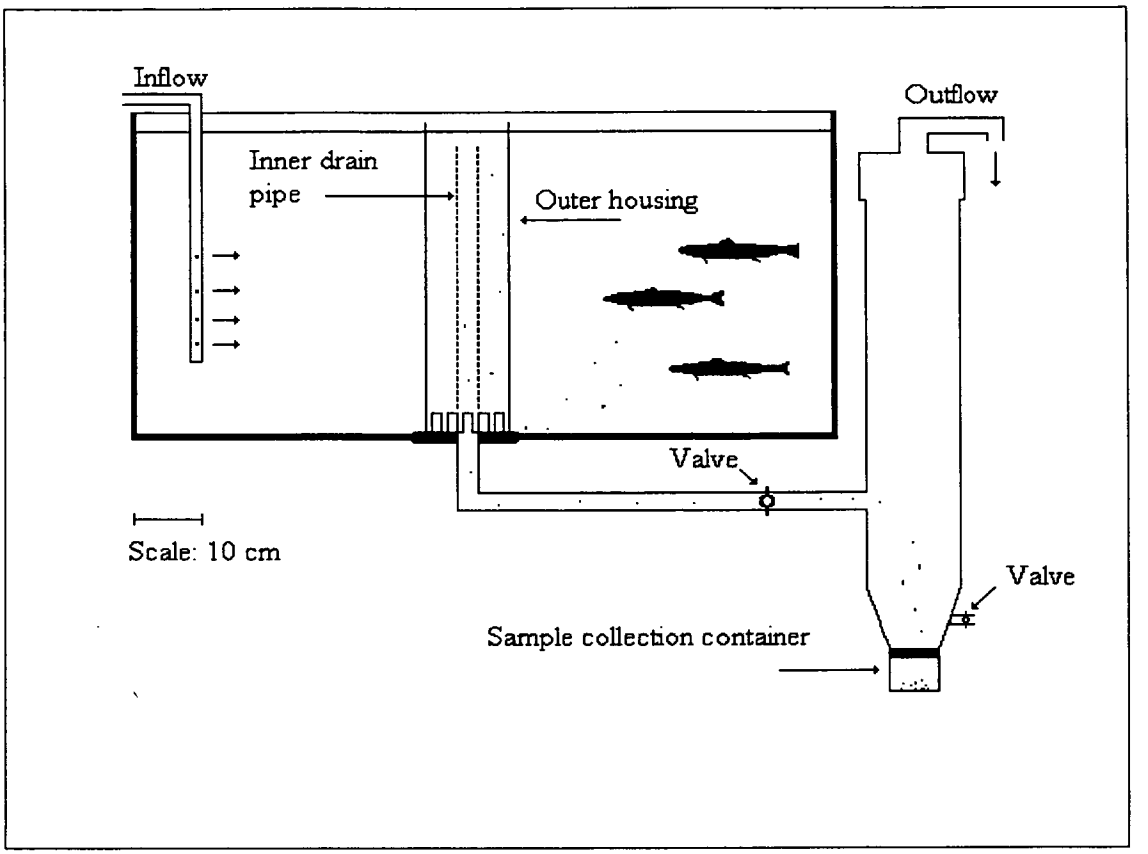

Figure 6.1 Diagram of an experimental tank with a faecal collector that is a modification of the Guelph system. The sterile sample collection container had a volume of 70 ml, and could be replaced as required. The 300-L tank had a flat bottom, and faecal material flowed into the outer housing, through holes in the bottom, and then down the inner drain pipe.

6.2.4.1 Sample decomposition and elemental content analyses

Approximately one gram of the freeze-dried faecal samples and samples of each experimental feed were wet-decomposed at 100 °C with 5 ml of nitric acid (Aristar Grade, 16M HNO_3). If required, an additional 5 ml of nitric acid was used in the decomposition of faecal samples. After decomposition the samples were made up to a volume of 50 ml with purified, de-ionised water. Samples were diluted a further 10-fold to improve the determination of highly concentrated minerals, such as phosphorus and calcium.

The samples were analysed for a range of minerals and trace elements using ICP-optical emission spectrophotometry (ICP-OES), including yttrium the internal marker. The analyses were performed at the Central Science Laboratory (University of Tasmania, Hobart, Tasmania, Australia). In addition to the internal quality controls samples used during ICP-OES analyses, samples of dogfish muscle tissue (DORM-2, from the National Research Council, Canada) were used as quality controls for the analytical procedures.

6.2.4.2 Apparent digestibility coefficients

The apparent digestibility coefficient (ADC) was determined according to:

$$ADC(\%) = 100 - \left[\frac{[M]feed \times [N]faeces}{[M]faeces \times [N]feed}\right] \times 100 \qquad [6.1]$$

(Maynard & Loosli, 1969) where [*M*] was the concentration, in mg kg^{-1}, of the digestibility marker, and [*N*] the concentration of the nutrient in mg kg^{-1}. The mineral and trace element digestibility for an experimental ingredient was determined using the following equation (Sugiura et al., 1998a):

$$ADCN_{ingr} = \left([N]_{com} \times ADCN_{com} - 0.7\,[N]_{ref} \times ADCN_{ref}\right) / \left(0.3 \times [N]_{ingr}\right) \qquad [6.2]$$

where $ADCN_{ingr}$ = the apparent digestibility coefficient (%) of the nutrient in the ingredient; $[N]_{com}$ = the concentration of the nutrient in the combined feed; $ADCN_{com}$ = the apparent digestibility coefficient of the nutrient in the combined feed; $[N]_{ref}$ = the concentration of the nutrient in the reference feed; $ADCN_{ref}$ = the apparent digestibility coefficient of the nutrient in the reference feed; and $[N]_{ingr}$ = the concentration of the nutrient in the ingredient.

In cases where the $ADCN_{ingr}$ was greater than 100% or negative the $ADCN_{ref}$ was recalculated assuming the $ADCN_{com}$ of the nutrient in the test ingredient was either 100% or 0% (Sugiura et al., 1998).

6.2.5 Statistical analyses

All statistical analyses were performed using SPSS v. 10.0 software (SPSS, 2000) and applied statistical methods described in Zar (1984) and Underwood (1981). For statistical analysis ingredient samples, experimental feed samples, faecal samples collected from one tank and feed intake from one tank were analysed. All ADC data

were tested for normality (Shapiro-Wilk), and, where applicable, ADC data were arcsine transformed with the following equation (Zar, 1984):

$$ADC' = \arcsin\sqrt{ADC} \qquad [6.3]$$

Some calculated ADC data were negative and it was not possible to arcsine transform these values. One blank sample, containing only the nitric acid used to decompose the feed and faecal samples, showed very high calcium (103.6 mg kg^{-1}) and phosphorus (73.1mg kg^{-1}) concentrations as compared to the remaining blanks and was not used in calculating mineral or trace element concentrations of the samples processed within that batch. Tukey's honestly significant difference test (Tukey's HSD) was used for multiple comparison of means for all data. Significance for all statistical tests was accepted at probability levels of 0.05 or less.

6.3 Results and discussion

All groups completely consumed the 2.0% body weight of feed provided throughout the period of the experiment and there were no significant differences ($P > 0.05$) in feed intake (69.3 ±0.42 g d^{-1}) between tanks. There were no mortalities or significant changes in temperature or water quality throughout the experiment.

6.3.1 ADC

6.3.1.1 Marker recovery

There were no significant differences ($P > 0.05$) in the percentage of yttrium recovered from each feed (Table 6.7). The recovery of yttrium was similar to those achieved in earlier experiments (Chapters 2, 4 and 5), close to 100%, and the slight variance observed may reflect a lack of thorough mixing or settling of the marker within the feed (Hillestad et al., 1999; Austreng et al., 2000). There was a significant difference between the yttrium content of the reference feed and the experimental feeds, refecting the 30% substitution of the experimental ingredients which contained no detectable concentrations of yttrium.

6.3.1.2 Macronutrient ADC

The REF feed had the highest level of digestible energy (ADC_{MJ}) and digestible crude protein (ADC_{cp}). However, a comparison of the three experimental feeds showed no significant differences ($P > 0.05$) for the ADC calculated for crude protein or energy calculated for all three of the experimental ingredients (Table 6.8). Based on these data alone the three plant ingredients were roughly equivalent in their usefulness as protein substitutes for fish meal in feed for Atlantic salmon, when comprising 30% of the total feed formulation. The ADC of crude protein in *L. albus* and *L. anagustifolius* (84% and 77%) was slightly lower than crude protein ADC values for *L. anagustifolius* [Gungurra] protein concentrate for Atlantic salmon (95%) (Carter & Hauler, 2000), dehulled, extruded *L. albus* for rainbow trout (96%)

Table 6.7 The mean (±SEM, $n = 3$) recovery of the digestibility marker yttrium oxide from the experimental feeds

Parameter	Feed				F value	*P*
	REF	ALB	ANA	SOY	(*df*=3)	
Expected yttrium (mg kg^{-1})	787.5	551.3	551.3	551.3		
Feed yttrium (mg kg^{-1})	834.02^{b} (17.02)	618.38^{a} (51.10)	551.44^{a} (2.84)	573.43^{a} (31.00)	51.99	<0.001
Yttrium recovery (%)	105.7 (2.16)	112.0 (9.26)	99.9 (0.51)	103.8 5.62)	2.508	ns

Means with the same superscript were not significantly different (Tukey's HSD).

Table 6.8 The effect of the experimental feeds on the mean (±SEM, n = 5) digestible energy, digestible crude protein, and ADC (%) of energy, crude protein and minerals

Parameter	Feed				F value	P
	REF	ALB	ANA	SOY	(df=3)	
Macronutrients						
Digestible energy (MJ kg^{-1} DM)	18.2 (0.1)	17.6 (0.1)	16.5 (0.1)	16.9 (0.0)	n/a	n/a
$ADCN_{MJ}$ (%)[1]		78.7 (0.2)	75.7 (0.3)	78.7 (0.7)	2.28	ns
Digestible crude protein (g kg^{-1} DM)	430.3 (0.1)	397.4 (0.2)	381.8 (7.7)	431.5 (1.1)	n/a	n/a
$ADCN_{CP}$ (%)[2]		84.0 (3.3)	77.1 (2.2)	81.2 (0.8)	5.71	ns
Minerals						
Ca	8.7^{a} (3.3)	11.4^{ab} (4.4)	20.5^{b} (1.4)	13.7^{ab} (5.0)	3.59	0.037
Fe	1.8^{a} (7.7)	17.7^{ab} (16.1)	17.9^{ab} (17.9)	29.0^{b} (24.1)	3.80	0.031
K	99.7 (0.0)	99.6 (0.0)	99.7 (0.3)	98.1 (0.1)	2.57	ns
Mg	37.9^{a} (2.1)	52.8^{bc} (2.8)	46.5^{ab} (5.7)	57.1^{c} (0.5)	12.48	<0.001
Na	94.3^{bc} (0.0)	95.6^{c} (0.0)	90.9^{a} (0.3)	92.4^{ab} (0.1)	15.67	<0.001
P	50.9^{a} (1.3)	54.9^{ab} (1.4)	59.1^{b} (0.4)	54.4^{ab} (1.2)	5.27	0.010
S	89.6 (0.6)	90.4 (0.2)	91.0 (0.1)	89.8 (1.1)	0.41	ns

Means with the same superscript were not significantly different (Tukey's HSD).

[1] The apparent digestibility coefficient (%) of energy for each ingredient

[2] The apparent digestibility coefficient (%) of crude protein for each ingredient

(Burel et al., 2000), and *L. anagustifolius* [Gungurra] meal in rainbow trout (79.2%) (Glencross et al., 2003). The SOY feed contained a greater concentration of crude protein but the digestibility of crude protein was equivalent to the other experimental feeds and comparable to digestible protein values for soy protein concentrates calculated in rainbow trout (Kaushik & Medale, 1994; Aksnes & Opstvedt, 1998).

6.3.1.3 Mineral and trace element ADC: experimental feeds

There were a number of significant differences in mineral and trace element ADC calculated for the experimental feeds (Table 6.8). The ADC for sodium in the REF feed was significantly higher than the other feeds, but the ADC for calcium, iron, and magnesium were lower. There were no significant differences ($P > 0.05$) in potassium or sulphur ADC for any of the feeds. The trace element ADC varied for magnesium, sodium, aluminium, boron, barium, cobalt, cadmium, manganese, nickel, silicon and zinc (Table 6.9). The REF feed had the lowest ADC for copper, molybdenum, and nickel and the highest ADC for boron. The ALB feed had the lowest ADC for aluminium, boron, and barium. The ANA feed had the lowest ADC for cobalt, manganese, and silicon, and the highest ADC for zinc. The SOY feed had the highest ADC for cadmium and nickel.

Table 6.9 The effect of the experimental feeds on the mean (±SEM, n = 5) ADC (%) of trace elements

Element	Feed				F value	P
	REF	ALB	ANA	SOY	(df=3)	
Al	-47.8^{b} (771.3)	-233.3^{a} (1288.3)	-37.7^{b} (110.2)	-71.1^{b} (670.3)	5.91	0.006
B	82.2^{c} (0.8)	74.0^{a} (0.2)	79.6^{bc} (0.8)	76.9^{ab} (0.3)	12.23	<0.001
Ba	-29.4^{b} (25.9)	-84.6^{a} (54.6)	-17.6^{b} (6.0)	-27.7^{b} (25.8)	16.36	<0.001
Cd	26.6^{a} (2.9)	19.3^{a} (2.4)	23.5^{a} (5.1)	39.4^{b} (1.8)	12.11	<0.001
Co	73.2^{b} (5.4)	75.0^{b} (3.1)	59.6^{a} (11.9)	81.4^{b} (1.4)	7.66	0.002
Cu	33.8^{a} (6.0)	72.3^{b} (1.5)	65.6^{b} (4.2)	68.9^{b} (4.3)	39.35	<0.001
Mn	16.6^{b} (42.8)	6.2^{ab} (6.9)	-12.7^{a} (11.1)	17.8^{b} (0.8)	6.44	0.005
Mo	24.6^{a} (1.9)	80.4^{b} (1.5)	81.5^{b} (8.7)	86.7^{b} (1.5)	126.70	<0.001
Ni	-20.4^{a} (2.8)	20.8^{b} (29.9)	33.8^{b} (3.5)	69.3^{c} (0.3)	75.27	<0.001
Si	81.2^{b} (0.1)	81.1^{ab} (0.0)	79.0^{a} (0.1)	81.8^{b} (0.4)	5.14	0.011
V	75.9 (0.7)	70.4 (0.8)	76.1 (0.9)	76.9 (4.6)	2.55	ns
Zn	31.2^{a} (8.4)	38.2^{a} (2.3)	50.5^{b} (1.1)	41.6^{ab} (2.5)	9.06	0.001

Means with the same superscript were not significantly different (Tukey's HSD).

6.3.1.4 Mineral and trace element ADC: experimental ingredients

Mineral and trace element ADC calculated for the ingredients substituted into the reference feed were mostly above 100% or negative, with very few providing results within the normal range (Tables 6.10 and 6.11). The soybean protein concentrate had significantly lower ADC for calcium, potassium, phosphorus and copper and significantly higher ADC for sodium, molybdenum and silicon than the other ingredients. The *Lupinus anagustifolius* had significantly higher ADC for calcium, boron, vanadium and zinc than the other ingredients and significantly lower ADC for manganese and silicon. The *Lupinus albus* had significantly higher ADC for potassium, magnesium and cobalt, and significantly lower ADC for boron and molybdenum than the other ingredients.

The number of the mineral and trace element ADC calculated for the experimental ingredients which fall outside the 0 – 100% range for each of the ingredients was large. Only in those few cases where the concentration of the element was equivalent that element in the basal feed did the calculated ADC fall in the normal range. Sugiura et al. (1998a) suggested that ADC values outside the 0 – 100% range indicate the assumption that the digestibility of minerals in the basal feed is constant and unaffected by the test ingredients is not justified. In cases where the digestibility of an ingredient was greater than 100% or less than zero it was assumed the ingredient had either an antagonistic and synergistic effect between minerals and compounds in the feed and ingredients. Therefore, the ADC for the reference feed was recalculated for those ingredients (Table 6.12), with the ADC set at either 100% or 0%, to estimate the effect of the ingredient on ADC for the REF feed (Sugiura et al., 1998).

Table 6.10 The mean (±SEM, n = 5) mineral ADC (%) calculated for each experimental ingredient

Element	Ingredient[†]			F value	P
	L. albus	*L. anagustifolius*	*Glycine max*	(df=3)	
Ca	223.37[ab] (419.52)	539.47[b] (160.30)	81.88[a] (93.02)	3.91	0.049
Fe	331.91 (274.38)	263.31 (231.28)	224.23 (128.78)	0.30	ns
K	124.47[c] (0.72)	113.99[b] (0.19)	77.58[a] (3.79)	609.96	<0.001
Mg	138.70[b] (29.99)	71.74[a] (27.63)	86.06[a] (5.45)	11.02	0.002
Na	3381.23[a] (135.67)	2491.96[a] (836.40)	12298.23[b] (1639.20)	129.56	<0.001
P	206.11[b] (65.39)	232.36[b] (35.62)	86.49[a] (26.91)	14.46	0.001
S	107.60[b] (9.44)	105.43[b] (5.42)	73.69[a] (11.51)	21.53	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

[†] Elements with mean ingredient ADC greater than 100% include a recalculation of the ADC for that element in the reference portion of that feed [shown in brackets].

Table 6.11 The mean (±SEM, $n = 5$) trace element ADC (%) calculated for each experimental ingredient

Element	Ingredient†			F value (df=3)	P
	L. albus	*L. anagustifolius*	*Glycine max*		
Al	-655.89 (434.08)	110.07 (428.41)	-818.98 (1412.04)	1.56	ns
B	113.17^{a} (6.56)	226.60^{c} (17.19)	133.19^{b} (6.82)	142.83	<0.001
Co	-32.83^{c} (39.25)	-2318.38^{b} (360.62)	-3183.70^{a} (411.81)	131.98	<0.001
Cu	319.32^{b} (22.73)	291.19^{b} (44.90)	115.73^{a} (16.50)	65.10	<0.001
Mn	4.54^{b} (7.09)	-41.42^{a} (24.43)	29.33^{b} (13.60)	23.23	<0.001
Mo	13.49^{a} (0.75)	57.98^{b} (8.37)	244.86^{c} (12.68)	976.36	<0.001
Ni	62.05^{a} (26.90)	114.75^{a} (10.93)	2257.46^{b} (45.80)	8003.13	<0.001
Si	-3935.73^{b} (115.95)	-6313.18^{a} (228.75)	-461.75^{c} (141.58)	1513.66	<0.001
V	3.36^{a} (2.18)	18.65^{b} (2.31)	5.51^{a} (2.29)	67.29	<0.001
Zn	47.82^{a} (21.08)	132.10^{b} (17.74)	78.79^{a} (25.53)	19.31	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

† Elements with mean ingredient ADC greater than 100% or less than 0% include a recalculation of the ADC for that element in the reference portion of that feed [shown in brackets].

Table 6.12 The effect of the experimental ingredients on the mineral and trace element ADC of the reference portion of the experimental feeds, for those minerals and trace element where ingredient ADC were less than 0% or greater than 100%.

Element	REF ADC (%)	Recalculated reference ADC (%) by ingredient[1]		
		L. albus	*L. anagustifolius*	*Glycine max*
Minerals				
Ca	**8.7**	10.9	19.9	**8.7**
Fe	**1.8**	9.85	8.0	15.7
K	**99.7**	104.3	102.1	**99.7**
Mg	**37.9**	47.07	**37.9**	**37.9**
Na	**94.3**	109.9	100.1	104.5
P	**50.9**	57.9	59.2	**50.9**
S	**89.6**	91.0	90.7	**89.6**
Trace elements				
Al	**-47.8**	-180.5	-47.0	-144.0
B	**82.2**	86.4	123.0	98.1
Co	**73.2**	68.8	33.6	53.8
Cu	**33.8**	108.7	73.6	43.0
Mn	**16.6**	**16.6**	-40.1	**16.6**
Mo	**24.6**	**24.6**	**24.6**	115.2
Ni	**-20.4**	**-20.4**	-14.6	107.8
Si	**81.2**	63.0	62.3	72.3
Zn	**31.2**	**31.2**	38.1	**31.2**

[1] Those ADC that were not greater than 100% or less than 0% were not recalculated, and the REF ADC retained (shown in **bold**).

The recalculated mineral ADC values for the reference portions of the experimental feeds indicated that *L. albus* increased the ADC of all minerals, *L. anagustifolius* increased the ADC for all minerals except magnesium and soybean increased the ADC of iron and sodium. The plant ingredients had less effect on trace elements and increased the ADC of copper and reduced the ADC of silicon and cobalt in the reference portion of all the experimental feeds. Soybean increased the ADC of molybdenum and nickel in the reference feed. *L. anagustifolius* decreased the ADC for manganese, but this may be an artefact of the significantly high manganese content of this ingredient which could have resulted in increased excretion. The presence of minerals in the water, homeostatic processes, excessive mineral content in the experimental ingredients and analytical variability could account for those cases where recalculated ADC in the reference portion of these feeds were greater than 100%, such as potassium, sodium, aluminium, copper, manganese, and nickel.

These synergistic and antagonist effects highlight the difficulties in determining the ADC of elements using the substitution method with ingredients that are so dissimilar from the reference feed. In some cases the variance in content of some minerals in the basal feed were similar to the total content of those minerals in the test ingredients, and this results in ADC calculations that are not accurate. An example is sodium where the concentration in the reference feed was $10,920 \pm 338$ mg kg^{-1}, and the test ingredients ranged from 21 – 122 mg kg^{-1}, resulting in the extreme ADC calculated for sodium in all the ingredients.

6.3.2 Feed factors affecting ADC

There are a number of reasons why the experimental ingredients significantly affected nutrient ADC. The soybean protein concentrate feed had higher ADC for iron, nickel, manganese, cobalt and zinc than the lupin meals. Extracted soybean products are known to increase plasma zinc when fed to larger Atlantic salmon kept in seawater (Olli et al., 1995), which may result from improved digestibility coefficients. It is unclear why the nickel ADC for the SOY feed was so high, though the nickel content was similar to the ALB feed. Feed is the common route of nickel exposure in trout (Dallinger & Kautaky, 1985), and processing residue from stainless steel or growing conditions of the soybean may provide a form of nickel that is more available in this ingredient. The highly negative ADC for manganese calculated for the lupin feeds is the result of high content of manganese in the faecal samples, as the concentration of this mineral is much higher in these feeds producing an increase in excretion levels. Therefore, it is unlikely the ADC for manganese is negative, but instead impacting on manganese regulatory systems and producing a false response (Sugiura et al., 1998a). In general, the plant ingredients provided higher ADC than the REF feed for magnesium, copper, molybdenum and nickel, and to a lesser extent calcium, iron and phosphorus. The results from the present study differ from those significantly lower ADC for calcium, magnesium, sodium and zinc in large Atlantic salmon fed soy protein concentrate (Storebakken et al., 1998a).

6.3.3 Additional factors effecting ADC

There area also a number of reasons, other that those related to the experimental ingredients, that could affect nutrient ADC. Leaching and solubility differences (Windell et al., 1978; Vandenberg & de la Noüe, 2001), the method of faecal collection (de la Noüe & Choubert, 1986; Fernandez et al., 1996), the type of digestibility marker (Kotb & Luckey, 1972; Austreng, 1978; Tacon & Rodrigues, 1984; Kabir et al., 1998; Morales et al., 1999; Austreng et al., 2000), and how the marker is added to the feed (Hillestad et al., 1999) can produce different ADC for macronutrients and minerals. Significant differences in the absorption of potassium, calcium, magnesium, zinc, sodium and iron have been attributed to genetics in Atlantic salmon, possibly connected to drinking rate, active absorption of nutrients, and the absorption or excretion mechanisms of elements (Thodesen et al., 2001). Net mineral absorption differs between species of salmon and there are positive correlations between intake and the apparent availability of potassium, sodium and zinc in salmonids (Sugiura et al., 1998a). Feed particle size is another factor that should be considered, although it has been shown to have no effect on phosphorus digestibility in rainbow trout (Zhu et al., 2001), it may have an effect on the digestibility of other minerals, possibly in relation to the effect of particle size on gastric empyting (Jobling, 1987). The experimental design used in the present experiment should have limited the effect of most of these factors, aside from the differences in leaching and solubility resulting from differences in the form of minerals available in the ingredients.

Antagonistic and synergistic effects from substances in the ingredients produce differences in the mineral and trace element ADC. These factors have been reported to negatively effect manganese availability from various sources of protein (Sugiura et al., 1998a), and a number of minerals and trace elements were affected by the experimental ingredients, altering the ADC of these element in the reference portion of these feed. There were a number of significant correlation coefficients between dietary mineral concentrations and the ADC calculated for other minerals. There were highly significant negative correlations between phosphorus, sodium and iron concentrations in the feeds and the ADC of calcium, iron, magnesium, and phosphorus (Table 6.13). There were no significant correlations coefficients ($P >$ 0.05) between any minerals and the ADC calculated for either sodium or sulphur. Of the six elements that showed significant correlations coefficients between concentration in the feed and ADC calculated, copper, molybdenum and magnesium were positive and iron, phosphorus and zinc negative (Table 6.14). The negative correlation coefficients between the concentrations of phosphorus and the digestibility coefficients of other minerals in these feeds, may be linked to phytate-mineral complexes in plant ingredients (Storebakken et al., 1998b; Vielma et al., 2000). Phytate from oilseeds, and its derivatives, can bind to dietary minerals, such as zinc, magnesium, copper, calcium, iron, manganese, molybdenum and cobalt, making them unavailable or partially available for absorption in a number of mammalian species (Maga, 1982).

Table 6.13 Correlation coefficients between mineral concentrations in the experimental feeds and calculated ADC (%) for each mineral

	Mineral concentration						
ADC	[Ca]	[Fe]	[K]	[Mg]	[Na]	[P]	[S]
Ca	-0.442	-0.475*	-0.490*	0.027	-0.470*	-0.504*	-0.423
Fe	-0.491*	-0.447*	-0.354	0.424	-0.561*	-0.515*	-0.400
K	0.083	0.026	-0.078	-0.554*	0.190	0.120	-0.027
Mg	-0.612**	-0.541*	-0.415	0.471*	-0.666**	-0.597**	-0.514*
Na	0.260	0.292	0.283	-0.339	0.384	0.400	0.176
P	-0.606**	-0.638**	-0.656**	-0.074	-0.599**	-0.639**	-0.606**
S	-0.205	-0.223	-0.245	-0.119	-0.182	-0.206	-0.224

Pearson correlation coefficient (n = 15); * significant at the 0.05 level; ** significant at the 0.001 level.

Table 6.14 Correlation between trace element ADC (%) and the concentration of that element in the experimental feeds

Element	Pearson correlation coefficient
Al	0.393
B	-0.188
Co	0.188
Cu	0.472*
Mn	-0.101
Mo	0.600**
Ni	0.118
Si	0.240
V	0.388
Zn	-0.531*

Pearson correlation (n = 15); * significant at the 0.05 level; ** significant at the 0.001 level.

The reduced digestibility of phosphorus with increasing dietary concentrations confirm similar observations in rainbow trout (Green et al., 2002) and Atlantic salmon (Sugiura et al., 1998a), and the transport of inorganic phosphorus in the intestine of rainbow trout is known to be regulated by dietary phosphorus and sodium dependent (Avila et al., 2000). The reduction in magnesium digestibility with increasing dietary calcium and phosphorus could account for the reduction in plasma magnesium observed by Vielma and Lall (1998) when feeding increased dietary phosphorus and calcium to Atlantic salmon. The concentrations of magnesium, copper, and molybdenum in the feed displayed significant positive correlation coefficients with their respective ADC, suggesting that the absorption mechanism of these elements may not have reached a saturation point. The lack of any significant correlations between any mineral concentrations and the ADC calculated for sodium and sulphur suggest that there were no interactions between these elements and others in their absorption pathways. Factorial modelling of the effect of graded substitution of ingredients on mineral ADC calculations could be used to identify ingredient interactions (Shearer, 1995).

A number of investigations have examined the effect of plant ingredients on phosphorus digestibility (Table 6.15). It is clear that there is some variety in the phosphorus ADC attributed to single species such as soybean, and possibly some differences between species of salmon regarding digestibility of phosphorus within a single ingredient. The phosphorus digestibility results of the lupins in the present

Table 6.15 The ADC (%) values of phosphorus, in order of increasing digestibility, in plant ingredient-based salmonid feeds from other investigations for rainbow trout (RT) and Atlantic salmon (AS).

Fish[2]	P ADC (%)	Ingredient[1]	Reference source
RT	-18.9	Soybean meal (heat processed)	(Riche & Brown, 1996)
RT	-13.4	Soybean meal (solvent extracted)	(Riche & Brown, 1996)
RT	4.8	Canola meal (30% crude protein)	(Riche & Brown, 1996)
RT	8.4	Soybean meal (raw, full fat)	(Riche & Brown, 1996)
RT	8.5	Corn gluten	(Sugiura et al., 1998)
RT	12.5	Soybean (extruded full-fat)	(Cheng & Hardy, 2003)
RT	19.0	Rice bran	(Lall, 1991)
RT	22.0	Soybean meal	(Sugiura et al., 1998)
RT	22.1	Peanut meal	(Riche & Brown, 1996)
RT	26.4	Rapeseed meal (solvent-extracted)	(Burel et al., 2000)
RT	30.7	Corn gluten meal	(Riche & Brown, 1996)
RT	41.8	Rapeseed meal (heat-treated)	(Burel et al., 2000)
RT	42.6	Extruded peas	(Burel et al., 2000)
RT	44.5	Soybean meal	(Vielma et al., 1998)
RT	47.0	Wheat flour	(Sugiura et al., 1998)
RT	52.1	Dent corn (low phytate)	(Sugiura et al., 1999)
RT	52.1	Dent corn (low phytate)	(Sugiura et al., 1999)
RT	52.3	Dent corn	(Sugiura et al., 1999)
RT	52.3	Dent corn	(Sugiura et al., 1999)
RT	53.1	Flint corn	(Sugiura et al., 1999)
RT	53.1	Flint corn	(Sugiura et al., 1999)
RT	53.2	Barely (low-phytate)	(Sugiura et al., 1999)
RT	53.3	Cotton seed meals	(Cheng & Hardy, 2002)
RT	54.0	Barley	(Sugiura et al., 1999)
RT	54.8	Flint corn (low phytate)	(Sugiura et al., 1999)
RT	54.8	Flint corn (low phytate)	(Sugiura et al., 1999)
RT	55.3	Wheat middling	(Sugiura et al., 1998)
RT	56.0	Soybean meal	(Sugiura et al., 2001)
RT	58.0	Wheat germ	(Lall, 1991)
RT	61.9	Extruded lupin	(Burel et al., 2000)
RT	74.7	Wheat gluten	(Sugiura et al., 1998)
AS	29.7	Soybean (protein concentrate)	(Storebakken et al., 1998b)
AS	32.0	Wheat middlings	(Lall, 1991)
AS	33.6	Soya protein concentrate	(Refstie et al., 1999)
AS	36.0	Soybean meal (de-hulled)	(Lall, 1991)
AS	37.7	Soybean meal (toasted extracted)	(Refstie et al., 1999)
AS	38.2	Isolated soybean protein	(Refstie et al., 1999)
AS	54.4	Soybean protein concentrate	This investigation
AS	54.9	White lupin flour	This investigation
AS	59.1	Sweet lupin flour	This investigation

[1] Ingredients were included in feeds at 30% of total feed.

study were similar to those obtained in other lupin research with rainbow trout (Burel et al., 2000). The results from this investigation were used to produce ingredient data sheets (see Appendix D, Table D.1) for future reference and submitted to the Australasian Livestock Feed Ingredient Database (SARDI, 2003).

6.4 Conclusions

The soybean protein concentrate and lupin meals used in this experiment were equivalent in the provision of digestible energy and crude protein, but provided significantly different concentrations of all minerals and most trace elements between species and cultivars. It was not possible to accurately calculate mineral and trace element ADC in feeds without purified ingredients. It may be necessary to use alternative methods of determining the ADC of minerals and trace element in ingredients that are so dissimilar in nutrient concentration from the reference feed. Mineral retention and other measures of bioavailability may be employed to determine the mineral provision of ingredients in aquafeeds.

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Chapter 7

The effects of high inclusion of lupin meal (*Lupinus albus* and *Lupinus anagustifolius* [Gungurru cultivar]), on growth, mineral and trace element digestibility, mineral retention and maximum voluntary feed intake in juvenile Atlantic salmon (*Salmo salar* L.)

Abstract

This experiment investigated the effects of replacing 44% of the protein provided by fish meal with two Australian lupin meals, *Lupinus albus* and *Lupinus anagustifolius* [Gungurru cultivar], on growth parameters, apparent digestibility coefficients (ADC), mineral retention and feed intake in juvenile Atlantic salmon (*Salmo salar*, L.). The *L. albus* feed contained significantly more magnesium, molybdenum, nickel and manganese than the *L. anagustifolius* feed. Both lupin species produced significantly better growth and feed efficiency ratios than the fish meal based control feed. The lupins differed significantly in the ADC of arsenic, cobalt and manganese. These differences in content and digestibility were reflected in the significantly higher carcass content of cobalt and manganese in those fish fed the *L. albus* feed. The lupin feeds significantly increased the retention (g kg^{-1}) of iron, sodium, phosphorus, cobalt, and zinc as compared to the control feed.

The experimental feeds significantly affected mean feed intake as measured by counting waste feed pellets. Therefore, maximum voluntary feed intake was measured with X-radiography at the conclusion of the growth experiment to compare the two methods and to assess the effect of changing feed pellet size. Significantly higher maximum voluntary feed intakes (≈ 25%) were obtained with X-radiography, but increasing pellet size had no effect on feed intake. X-radiographic measurements of maximum voluntary feed intake could be used as a correction factor for feed intake measured by counting waste feed pellets.

Keywords: Aquaculture feeds, maximum voluntary feed intake, lupin, mineral digestibility, Atlantic salmon

7.1 Introduction

There is a growing need to identify and evaluate high protein alternatives to fish meal. Fish meal is the main component of aquafeeds used for Atlantic salmon production and the demands for fish meal to meet increases in aquaculture production are increasing (FAO, 2002) Fish meal can be costly, and is subject to price variation. Reducing the need for fish meal in salmon feed can improve the sustainability of intensive aquaculture production (Naylor et al., 2000) Therefore, reducing and/or replacing the fish meal portion of finfish aquafeed has been the subject of considerable research, with much of the recent focus on high-protein plant ingredients, including lupins (Hughes, 1988, 1991; Burel et al., 1998; Burel et al., 2000; Carter & Hauler, 2000; Refstie et al., 2000; Farhangi & Carter, 2001b). Reducing the fish meal portion of a trout feed with 30% extruded lupin had no effect on protein digestibility compared to the high quality fish meal control in rainbow trout (Burel et al., 2000). Higher levels of lupin inclusion (33% - 40%, limited growth in Atlantic salmon (Carter & Hauler, 2000) and rainbow trout (Farhangi & Carter, 2001a), but the reasons for the limitations in growth were not clear. It was also possible that the availability of minerals and trace elements from these ingredients were a limiting factor in growth when compared to fish meal based feed. Atlantic salmon can maintain rapid somatic growth while developing subclinical deficiencies in calcium, magnesium and phosphorus (Åsgård & Shearer, 1997; Baeverfjord et al., 1998; Storebakken et al., 2000). Lupins contain anti-nutritional factors, such as oligosaccharides and non-starch polysaccharide carbohydrates which interfere with the digestion of macronutrients (Glencross et al., 2003). It was necessary to determine if the minerals and trace elements present in *L. albus* and *L. anagustifolius*

were readily digestible and available to salmon, and if there were any antagonistic effects of minerals or compounds within the plant material that affect the availability of elements within the diet. It is important to verify the utilization of these essential elements in protein replacement studies (Storebakken et al., 2000).

The inclusion of novel ingredients often affects maximum voluntary feed intake (Refstie et al., 1997; Wybourne & Carter, 1998). The maximum voluntary feed intake of fish clearly has a major affect on growth rate, feed efficiency ratio (FER) and mineral status. It is important to determine the most effective means of observing the maximum voluntary feed intake under experimental conditions, and identify if an ingredient alters maximum voluntary feed intake. The most common methods of monitoring feed intake are direct observation of feeding or monitoring waste feed (Helland et al., 1996), methods such as the use of on-demand feeders or X-radiography may be appropriate (McCarthy et al., 1993; Carter et al., 1995; Jobling et al., 1995).

The aim of this experiment was to identify the effect on growth, mineral and trace element ADC, mineral and trace element retention of substituting 44% of the protein derived from fish meal with two Australian lupin meals: measurements were made on the mineral content of each feed and ingredient; experimental feed; feed intake; ADC for minerals and trace elements; growth; carcass mineral concentrations and mineral and trace element retention; and the retention of the digestible fraction of the minerals and trace elements. Additional aims were to compare common methods of determining the maximum voluntary feed intake, by counting waste feed and with X-radiography, to assess any effects on feed intake and feed intake measurements due to

a change in pellet size. Non-invasive methods were chosen to minimise the impact on the feeding patterns of the fish, and the X-radiographic measurements were made at the end of the experiment to avoid any effects on maximum voluntary feed intake and growth.

7.2 Materials and methods

7.2.1 Feeds

Three different feeds were formulated (Table 7.1): a control (CON), based on a reference salmon feed; and two feeds with 44% of the protein provided by either white lupin (*Lupinus albus*) meal (ALB) or Australian sweet lupin (*Lupinus anagustifolius* [Gungurru cultivar]) meal (ANA). The lupin meal was milled from pre-cleaned, dehulled lupin seed, so that 100% of the meal passed through a 2.55 mm screen. The feeds were formulated based on the digestible energy and crude protein determined for each plant ingredient prior to the growth experiment (Chapter 6), and included a mineral and vitamin mix (Table 7.2) formulated to meet all the nutritional requirements of Atlantic salmon or salmonids (NCR, 1993). The fish oil content of the ANA feed was adjusted to provide isoenergetic feeds, as this ingredient had a greater energy content than the other lupin. The CON feed was balanced to match the lupin-based feeds with the addition of starch.

Each feed was pelleted at room temperature using a California Laboratory Pellet Mill (CL-2 laboratory pellet mill, California Pellet Mill Co., San Francisco, U.S.A.). Two sizes of feed pellet, 1.5 mm and 3.0 mm, were used. Maximum voluntary feed intake

Table 7.1 Ingredient and chemical composition of the experimental feeds

Ingredient composition (g kg^{-1})	Source[1]	Feed[2] CON	ALB	ANA
Fish meal (Peruvian)	Skretting	628	350	350
Albus lupin meal	M.C. Croker	0	476	0
Anagustifolius lupin meal	M.C. Croker	0	0	496
Fish oil (Peruvian)	Skretting	124	125	106
Gel-Starch	Sigma-Aldrich	208	0	0
CMC binder	Sigma-Aldrich	9	9	9
Phenylalanine	Musashi	0	5	4
DL Methionine	BASF	0	4	4
Yttrium oxide (Y_2O_3)	Sigma-Aldrich	1	1	1
Mineral mix	See Table 7.2	30	30	30
Chemical compositions (g kg^{-1})				
Dry Matter		886	911	940
Crude protein		433	443	433
Gross energy (MJ kg^{-1} DM)		18.0	17.5	18.2

[1]Ingredients from Skretting and Gibson's were sourced from Cambridge, Tasmania; lupins ingredients from M.C. Croker, Cootamundra, New South Wales; Sigma-Aldrich products from Castle Hill, NSW; phenylalanine from Musashi, Victoria; and DL methionine from BASF, Melbourne, Victoria.

[2]CON: composed of 62.8% fish meal; ALB: composed of 47.6% *Lupinus albus* meal; ANA: composed of 49.6% *Lupinus anagustifolius* [Gungurru cultivar] meal.

Table 7.2 Mineral and vitamin supplementation mix added to the experimental feeds

Mineral and trace element supplements	mg kg^{-1}
Potassium phosphate dibasic (K_2HPO_4)	20000.00
Calcium carbonate ($CaCO_3$)	5000.00
Sodium chloride (NaCl)	2000.00
Magnesium carbonate ($MgCO_3$)	700.00
Ferrous sulphate ($FeSO_4$-$7H_2O$)	200.00
Zinc sulphate ($ZnSO_4$-$7H_2O$)	75.00
Manganous sulphate (MnSO4-4H2O)	80.00
Cupric sulphate ($CuSO_4$-$5H_2O$)	23.57
Cobalt sulphate ($CoSO_4$-$7H_2O$)	9.53
Potassium iodide (KI)	1.44
Sodium selenate (Na_2SeO_3)	0.66
Vitamin supplements	
Choline chloride	1333.33
myo-Inositol	300.00
DL alpha tocopherol acetate	200.00
Stay-C® (L-ascorbyl 2 polyphosphate)	50.00
Calcium D-pantothenate	21.73
Nicotinic acid	10.00
Retinol acetate (500000 IU/g)	4.80
Riboflavin	4.00
Pyridoxine HCl	3.66
Vitamin D_3 powder (400000 IU/g)	6.00
Menadone sodium bisulphate	2.00
Thiamin HCl	1.12
Folate	1.00
d-Biotin	0.15
Vitamin B_{12}	0.01

All mineral and trace element supplements were sourced from Sigma-Aldrich (Castle Hill, NSW), except for Stay-C® which was sourced from Roche (Frenchs Forest, NSW).

was measured by counting pellets, and the 3.0 mm pellet was used for radiographic measurement (see below).

The different sized feeds were processed from the same batch of feed, but pelleted on different days (batches of feed were stored in a cold room at 2.7 °C between pelleting). After finishing an experimental feed the pellet mill was cleaned of residual feed. X-ray opaque Ballotini glass beads (no. 8.5, 400-450 μm, Jencons Scientific Ltd., Leighton Buzzard, UK) were included in the 3.0 mm feed at a concentration of 2% in the 3.0 mm cold pressed feed (Carter et al., 1994). The X-ray feed was mixed and pelleted the same time as the larger feed. All pelleted feeds were oven-dried at 40 °C for over 24 h, and stored in a cold room at 2.7 °C.

7.2.2 Fish, experimental systems and faecal collection

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). The twelve 300-L, cylindrical tanks comprising the experimental system were connected to a temperature-regulated (14.7 ± 0.76 °C), 2000-l reservoir. Photoperiod was not controlled. Water was treated through physical and biological filters, with a continuous replacement of approximately 10% per day from the municipal water supply. Replacement water entered the system though an activated charcoal filter, to reduce mineral fluctuations. The system supplied filtered water to each tank at an average flow rate of 6 l min^{-1}. Water parameters (dissolved oxygen, oxygen saturation, chlorine, pH, ammonia, nitrate and nitrite) were monitored

to ensure water quality remained within limits recommended for Atlantic salmon (Wedemeyer, 1996).

Atlantic salmon parr (17.60 ± 0.94 g) were randomly allocated between each of the twelve tanks until a total of 60 fish were in each tank. One fish from each tank was removed randomly at the end of the distribution, euthanased and frozen (-4 °C) for later carcass analysis. Four replicate tanks were fed twice per day (09:00 and 17:00) by hand one of the three feeds. The daily ration was set at 2.5% of the tank biomass, and fish were weighed on days 21, 44 and 64 to adjust this amount. At day 93 all fish were removed, anaesthetised (benzocaine 30 mg l^{-1}), weighed and X-rayed, after a normal feeding routine (see X-radiography section below).

Fish faeces were collected using a modification of the Guelph design (Cho & Slinger, 1979), as described by Carter and Hauler (2000) (see Chapter 6, Fig. 6.1). Faecal samples were taken over an 18-h period starting after the last feeding of the 70th day of the growth experiment, and frozen immediately after collection and stored at -4 ° C. The entire set were freeze-dried as a unit at -10 to -12 ° C, and -100 to -110 kPa pressure, until all samples reached a constant weight.

Intake of each tank was monitored by counting waste feed recovered from baskets placed at the water outlets (Helland et al., 1996), at each feeding, every 5 days throughout the experiment. Pellets were counted after they ceased appearing in the baskets, normally 45 min after feeding. Observations were conducted after the morning and afternoon feedings. The number of pellets was multiplied by the mean pellet weight for that feed size to determine the amount of feed wastage, and

subtracted from the amount fed to provide the maximum voluntary feed intake for a tank.

7.2.3 Maximum voluntary feed intake

After the growth experiment maximum voluntary feed intake was determined, starting from day 64, to observe any possible effects of the high inclusion of lupins. Each tank was fed to satiation twice a day. The two methods of determining maximum voluntary feed intake employed were directly counting pellets (as described above), and X-radiography (details as in Carter et al. 1994). Two sizes of feed pellets (1.5 mm and 3.0 mm) were observed with the counting method, and one (3.0 mm) with the X-radiographic method. The size of the feed pellet was changed in the experiment to observe the effect of increasing pellet size on determination of maximum voluntary feed intake level.

7.2.4 X-radiography

The X-ray feed was provided as normal at the morning and afternoon feedings. All the fish from two of the four tank replicates for a feed treatment, determined randomly, were removed after the morning feed, anesthetised and X-rayed. The remaining replicates were X-rayed after the afternoon feeding. The X-rays, taken on Kodak Industrex AA400 film with a TR80/20 portable X-ray unit (Todd Research Ltd, Essex, UK), were processed immediately, according to the manufacturer's

instructions. The maximum voluntary feed intake was calculated by counting the number of beads in the digestive tract of each fish and applying feed calibration factors (Figure 7.1; CON: = 45.3 beads g^{-1} feed; ALB: 53.2 beads g^{-1} feed; ANA: = 39.8 beads g^{-1} feed). It was possible to identify those beads located in the stomach and those in the digestive tract beyond the stomach for comparative purposes.

7.2.5 Analytical procedures

Standard methods were used to determine dry matter (oven dried at 100 °C to a constant weight), gross energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid) and nitrogen (Kjeldahl using selenium catalyst).

7.2.5.1 Sample decomposition and analyses for mineral content

Approximately one gram of the freeze-dried faecal samples and samples of each experimental feed were wet-decomposed at 100 °C with 10 ml of concentrated nitric acid (Aristar Grade, 16 M HNO_3), then left to cool. After decomposition the samples were made up to a volume of 50 ml with purified, de-ionised water. The samples were analysed for yttrium oxide, and a full range of minerals and trace elements using ICP-optical emission spectrophotometry conducted at Central Science Laboratory (University of Tasmania, Hobart, Tasmania, Australia). Samples were diluted a further 10-fold to improve the determination of highly concentrated minerals, such as phosphorus and calcium.

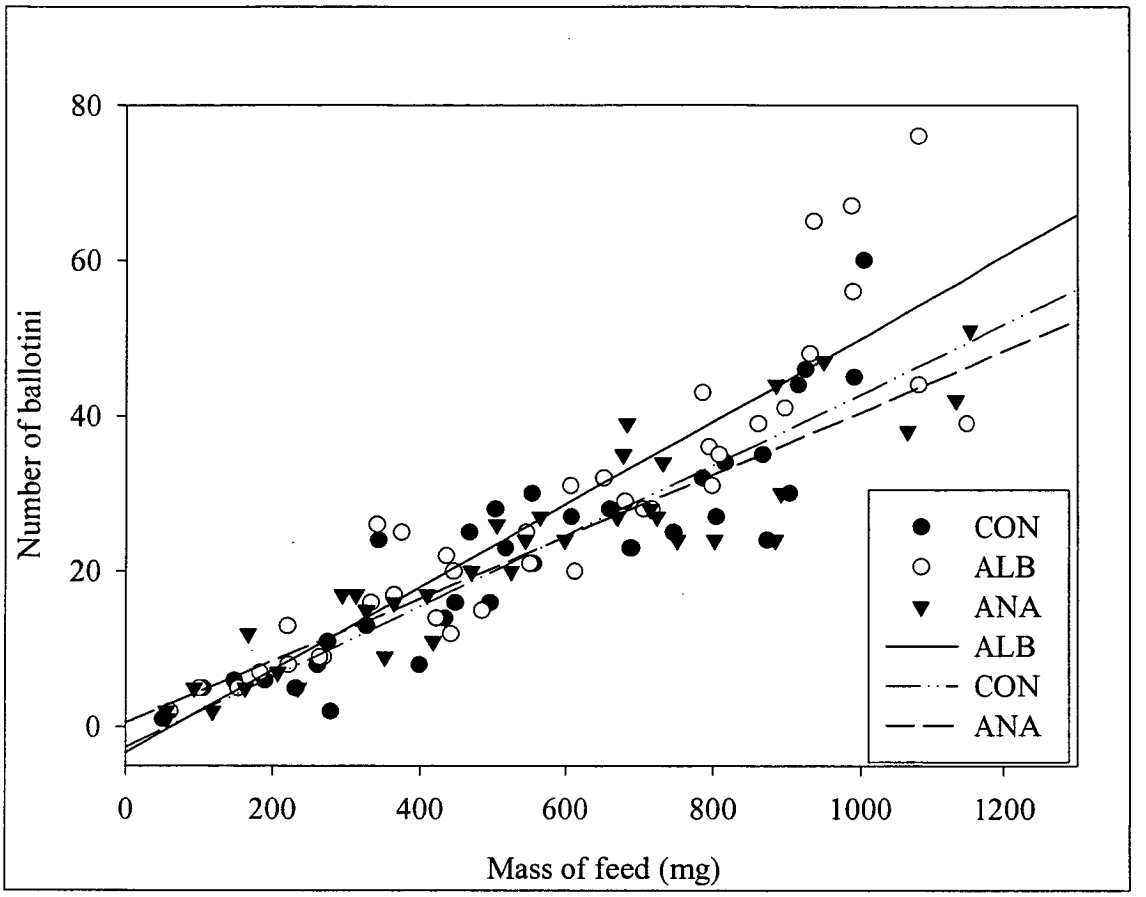

Figure 7.1 The number of X-ray opaque beads in a given mass of the experimental feeds: CON feed (mg) = 0.0453 beads - 2.6176, ($r^2 = 0.808$, $n = 39$, $P > 0.001$); ALB feed (mg) = 0.0532 beads - 3.3195, $r^2 = 0.802$, $n = 40$, $P > 0.001$); and ANA feed (mg) = 0.0398 beads + 0.5429, ($r^2 = 0.802$, $n = 40$, $P > 0.001$).

7.2.5.2 Apparent digestibility coefficients and retention

The apparent digestibility coefficient (ADC) was determined according to:

$$ADC(\%) = 100 - \left[\frac{[M]feed \times [N]faeces}{[M]faeces \times [N]feed}\right] \times 100 \qquad [7.1]$$

(Maynard & Loosli, 1969) where $[M]$ is the concentration (mg kg^{-1}), of the digestibility marker, and $[N]$ the concentration of the nutrient (mg kg^{-1}).

The apparent retention (%) was determined according to:

$$Apparent\ retention = 100 \times \left[\frac{(BW_f \times [N]_f) - (BW_i \times [N]_i)}{(Feed \times [N]_{Feed})}\right] \qquad [7.2]$$

(Storebakken et al., 1998b) where BW_f is the final body weight of the fish, $[N]_f$ is the concentration of nutrient (mg $fish^{-1}$) in the carcass taken on the final day of the experiment; BW_i is the initial body weight of the fish; $[N]_i$ is the concentration of nutrient in the carcasses taken at the start of the experiment (mg $fish^{-1}$); Feed is the total amount of feed consumed by the fish over the period of the experiment; and $[N]_{Feed}$ is the concentration (mg) of the nutrient provided to the fish over that time. The initial values were the average of 4 samples of 3 pooled fish from the 12 fish collected at the beginning of the experiment, and the final values were tank means from 3 salmon per tank collected on day 64. The body weights and feed intakes were mean values from each tank. Retention was calculated in the same way for minerals

(Ca, Fe, Mg, Na, P and S) and trace elements (B, Cd, Co, Cu, Mn, Mo, Ni, Y and Zn). The apparent retention of digested nutrients was calculated according to:

$$Apparent\ retention\ of\ digested\ nutrients = 100 \times Apparent\ retention \times ADC \quad [7.3]$$

(Storebakken et al., 1998b), where the apparent retention and ADC were those calculated from mean values for each tank.

7.2.6 Statistical analyses

All statistical analyses were performed using SPSS v. 10.0 software (SPSS, 2000) following the statistical methods described in Zar (1984) and Underwood (1981). For statistical analysis of growth and ADC the experimental unit was the tank. All ADC data were tested for normality (Shapiro-Wilk), and, where applicable, ADC (%) data was arcsine transformed with the following equation (Zar, 1984):

$$ADC' = \arcsin\sqrt{ADC} \quad [7.4]$$

All comparisons of feed intake, , fish weights, weight gain, cumulative SGR, FER, survival, ADC (%), feed and carcass mineral and trace element concentrations, mineral and macronutrient retention, retention of digestible minerals and trace elements, were all analysed using one-way analyses of variance with the experimental feed treatment as the fixed factor. The effect of pellet size and method of measuring maximum voluntary feed intake were also assessed using a one-way analysis of

variance with pellet size and method of measurement as the respective fixed factors. Tukey's honestly significant difference test (Tukey's HSD) was used for multiple comparison of means for all data, at a significance level of $P < 0.05$. Significance for all statistical tests was accepted at probability levels of 0.05 or less. One faecal sample collected from the CON feed was identified as an outlier, as it contained approximately $1/5^{th}$ the concentration of yttrium and other mineral and trace element concentrations of the other faecal samples. This sample was not included in any statistical calculations. Some calculated ADC data were negative, resulting from greater concentrations of an element in the faeces than the feed, and these values were not arc-sine transformed. Pearson correlation coefficients were calculated to compare the concentrations of elements in the tissue samples with the calculated ADC (%) and the concentrations of those elements in the feeds.

7.3 Results

7.3.1 Part 1: The growth trial

7.3.1.1 Feed parameters

The ALB and ANA feeds had significantly less calcium, iron, sodium and phosphorus and more potassium than the control feed (Table 7.3). The ANA feed had a significantly higher magnesium content that the other two feeds. The manganese content of the ALB feed was approximately 50 times greater than the control feed and 10 times greater than the ANA feed (Table 7.4). The concentrations of molybdenum,

Table 7.3 Mean (±SEM, n = 3) mineral concentrations (mg kg^{-1}) of the experimental feeds

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
Ca	29882.44[b] (1379.68)	20170.42[a] (1660.68)	18592.80[a] (1435.19)	50.05	<0.001
Fe	386.65[b] (81.35)	150.91[a] (8.56)	198.68[a] (46.96)	15.71	0.004
K	15727.46[a] (436.20)	19268.31[b] (639.75)	19105.55[b] (613.68)	36.84	<0.001
Mg	2234.75[a] (14.61)	2192.78[a] (7.00)	2429.66[b] (94.45)	15.66	0.004
Na	8317.88[b] (20.83)	6011.27[a] (177.83)	5702.73[a] (189.78)	270.03	<0.001
P	22179.16[b] (398.02)	17234.08[a] (1349.56)	16150.05[a] (753.87)	36.49	<0.001
S	33644.76 (2924.39)	34812.15 (2215.13)	34395.14 (3485.65)	0.12	ns

Means with the same superscript were not significantly different (Tukey's HSD).

Table 7.4 Mean (±SEM, $n = 3$) trace element concentrations (mg kg^{-1}) of the experimental feeds

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
Al	41.75 (2.14)	26.62 (5.55)	37.32 (19.76)	1.279	ns
As	3.97 (0.45)	2.19 (1.42)	1.95 (0.89)	3.624	ns
B	10.58^{a} (0.58)	17.59^{b} (1.39)	19.06^{b} (0.34)	77.801	<0.001
Cd	1.31^{b} (0.09)	0.96^{a} (0.04)	0.95^{a} (0.10)	20.303	.002
Co	2.08 (1.71)	2.48 (0.18)	0.88 (0.13)	2.084	ns
Cr	1.19 (0.14)	0.74 (0.31)	1.06 (0.21)	2.996	ns
Cu	30.71^{a} (1.27)	34.90^{b} (0.57)	34.48^{b} (1.71)	9.853	.013
Mn	41.87^{a} (2.24)	2039.30^{c} (73.08)	204.17^{b} (5.96)	2058.125	<0.001
Mo	0.89^{a} (0.35)	3.25^{c} (0.35)	2.43^{b} (0.08)	50.688	<0.001
Ni	0.22^{a} (0.06)	1.99^{c} (0.02)	1.40^{b} (0.16)	247.525	<0.001
Pb	1.90 (0.45)	3.06 (0.23)	1.69 (0.83)	5.171	ns
Se	0.78 (0.67)	2.24 (0.31)	2.36 (1.18)	3.550	ns
Y	10.93 (6.76)	-6.90 (4.49)	7.91 (3.69)	1.936	ns
Zn	-8.73 (0.90)	-9.74 (0.85)	-8.70 (1.43)	1.394	ns

Means with the same superscript were not significantly different (Tukey's HSD).

nickel and boron in the ALB and ANA feeds were 2 – 3 times greater than the control feed. Whereas the control feed had the greatest concentrations of cadmium.

7.3.1.2 Feed intake

There were no significant differences ($P > 0.05$) in feed intake until day 55, at which point the feed intake of the CON feed was significantly less than the other two feeds (Figure 7.2). The feed intake for those fish fed CON then fell throughout the next 10 days. The feed intakes were generally lower for CON than the lupin-based feeds, which increased over time.

7.3.1.3 Growth performance

There were no significant differences ($P > 0.05$) in overall survival between the feeds. The fish fed ALB and ANA consumed more total feed, had higher cumulative specific growth rates and better feed efficiency ratios than those fish fed the control feed (Table 7.5). Fish fed CON had significantly lower mean weights on day 44 ($F = 8.98$, $df = 2$, $P = 0.007$), day 64 ($F = 18.11$, $df = 2$, $P = 0.001$) and day 92 ($F = 10.56$, $df = 2$, $P = 0.004$) compared with fish fed ALB and ANA (Figure 7.3). There was a decline in specific growth rates over the course of the experiment (Figure 7.4), with fish fed the CON feed declining significantly more than the other treatments from days 21-44.

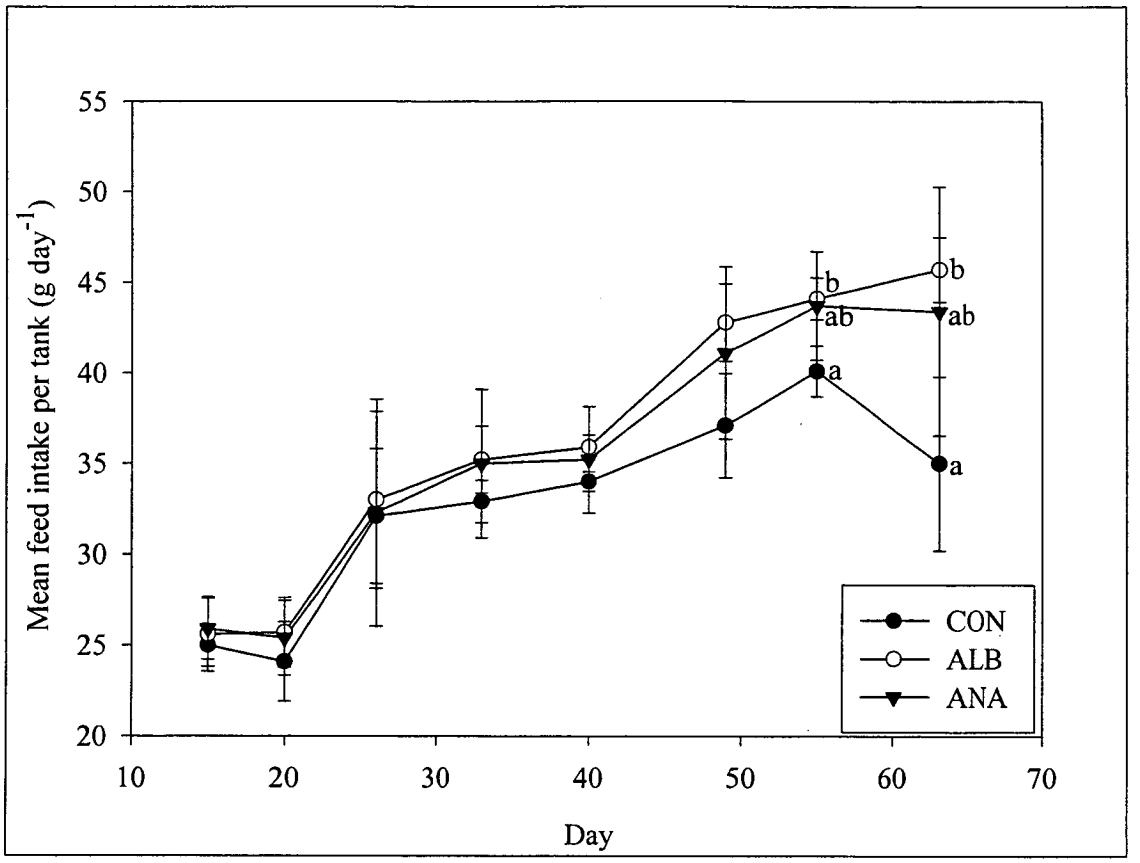

Figure 7.2 The effect of the experimental feeds on mean (±SEM, $n = 4$) feed intake per tank, as determined by collecting uneaten feed. Means with different superscripts were significantly different (Tukey's HSD). Comparison of means was by one way analysis of variance (Day 55, F = 4.88, $P = 0.037$; Day 63, F = 5.17, $P = 0.032$).

Table 7.5 The initial BW and the effect of the experimental feeds on the mean (±SEM, $n = 4$) weight gain, cumulative SGR, total feed consumption, FER, overall survival and faecal crude protein of Atlantic salmon

Parameter	Unit	Feed			F value	*P*
		CON	ALB	ANA	(*df*=3)	
Initial BW	(g)	17.48 (0.26)	17.83 (1.34)	17.59 (1.15)	0.11	ns
Weight gain	(g)	20.28^{a} (1.00)	27.87^{b} (2.19)	28.00^{b} (1.37)	33.98	<0.001
Cumulative SGR[1]	(% d^{-1})	1.17^{a} (0.05)	1.33^{b} (0.09)	1.38^{b} (0.04)	12.31	0.003
Total feed consumption[2]	(kg)	1.78^{a} (0.04)	2.03^{b} (0.06)	2.05^{b} (0.20)	5.89	0.023
FER[3]	(g g^{-1})	0.46^{a} (0.03)	0.59^{b} (0.04)	0.59^{b} (0.04)	16.84	0.001
Overall survival	(%)	98.8	99.2	100.0	0.535	ns
Faecal crude protein	(%)	43.7^{b} (4.3)	20.1^{a} (4.6)	15.2^{a} (3.9)	49.98	<0.001

Means with the same superscript were not significantly different (Tukey's HSD).

[1] The cumulative specific growth rate was calculated using fish weights taken from day 1 and day 64.

[2] Total feed consumption was the sum of all feed intake estimates for each experimental feed. Feed intake estimates were calculated by subtracting waste feed from total feed provided.

[3] Feed efficiency ratio = (total weight gain (g) / total feed consumption (g DM)).

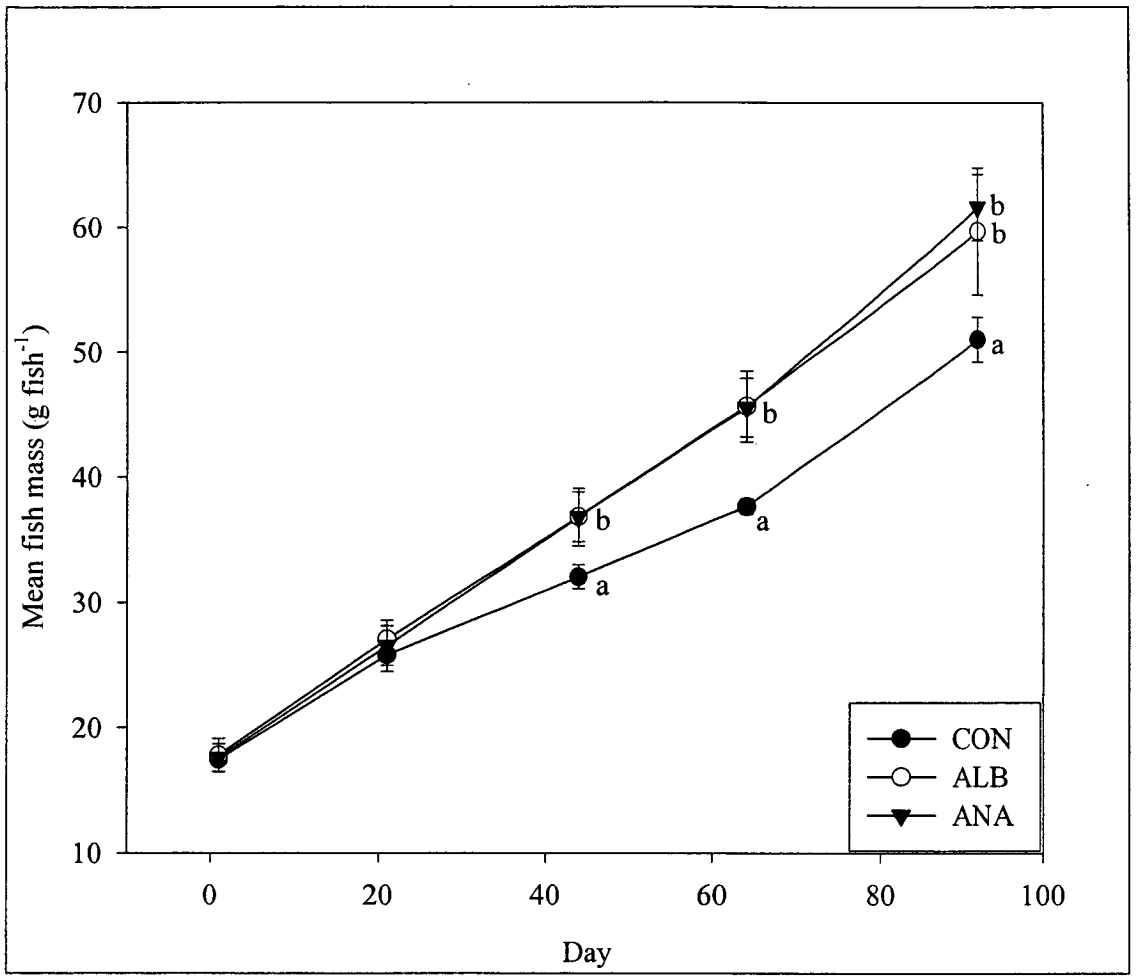

Figure 7.3 The effect of the experimental feeds on mean (±SEM, $n = 4$) fish weight (g $fish^{-1}$) day 1 to day 92. Means fish weights on day 1 and day 21 showed no significant differences, but from day 44 onward the mean weight of fish fed CON were significant lower (Tukey's HSD) than those fed ALB or ANA.

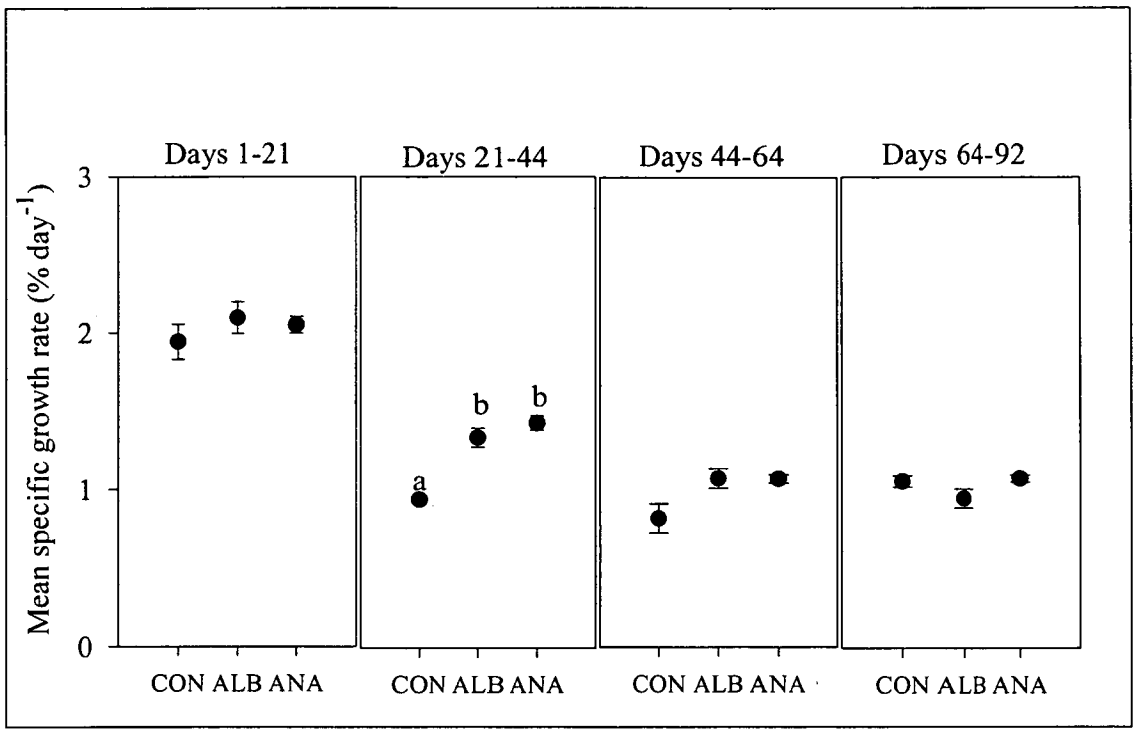

Figure 7.4 The effect of the experimental feeds on mean (±SEM, $n = 4$) specific growth rates (% day^{-1}), calculated over four time periods. The only significant difference was seen for the mean specific growth rates calculated from day 21- 44, where the mean specific growth rates for fish fed the CON feed were significantly lower (Tukey's HSD) than those fed ALB or ANA.

7.3.1.4 ADC

The CON feed had significantly lower ADC for crude protein than the ALB and ANA feeds, but all three were equal with respect to the digestibility of energy (Table 7.6). The ADC of potassium, magnesium, phosphorus and sulphur was significantly greater in the ANA and ALB feeds, than in the CON feed, which had higher ADC for iron; there were no differences in calcium or sodium ADC (Table 7.6). Almost all the trace elements analysed were significantly different. The ANA and ALB feeds provided higher ADC for copper, molybdenum, cobalt and manganese than the control feed (Table 7.7).

7.3.1.5 Carcass mineral and trace element content

There were no differences in the crude protein or mineral content of the fish carcasses (Table 7.8) taken at the end of the experiment, but there were significant differences in the content cobalt and manganese in the carcass samples (Table 7.9).

Table 7.6 The effect of the experimental feeds on mean (±SEM, $n = 4$) digestible crude protein (g kg^{-1} DM), digestible energy (MJ kg^{-1} DM) and on mineral ADC values (%) in Atlantic salmon

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
Crude protein	276[a]	396[b]	392[b]	129.60	<0.001
	(29)	(15)	(13)		
Energy	13.7	13.7	13.7	0.01	ns
	(0.1)	(0.0)	(0.1)		
ADC (%)					
Ca	11.90	26.44	14.54	2.36	ns
	(45.79)	(1.65)	(6.79)		
Fe	10.32[b]	-57.29[a]	-23.81[ab]	14.27	0.002
	(9.20)	(54.41)	(33.14)		
K	91.29[a]	98.61[b]	99.24[b]	76.23	<0.001
	(0.33)	(0.11)	(0.01)		
Mg	58.17[a]	72.55[b]	64.87[ab]	6.92	0.018
	(0.40)	(2.13)	(6.35)		
Na	80.92	87.24	82.30	0.36	ns
	(2.40)	(10.54)	(26.02)		
P	45.17[a]	60.75[b]	58.87[b]	9.96	0.007
	(10.54)	(0.26)	(2.42)		
S	87.79[a]	92.86[b]	91.69[b]	9.49	0.008
	(0.81)	(0.28)	(0.14)		

Mean ADC for each feed were calculated from three replicates for CON and four replicates for ANA and ALB. Means with the same superscript were not significantly different (Tukey's HSD).

Table 7.7 The effect of the experimental feeds on mean (±SEM, $n = 4$) trace element ADC (%) in Atlantic salmon

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
Al	-209.92 (2155.62)	-267.41 (3586.01)	-196.40 (2240.59)	0.27	ns
As	43.72^{b} (47.83)	59.30^{b} (14.07)	18.96^{a} (6.13)	12.47	0.003
B	50.59^{a} (15.92)	65.69^{b} (3.41)	67.57^{b} (1.57)	7.32	0.016
Cd	55.34^{a} (2.51)	68.49^{b} (1.48)	68.15^{b} (0.57)	18.55	0.001
Co	44.49^{ab} (1.84)	74.15^{b} (22.68)	25.97^{a} (118.91)	5.51	0.031
Cr	-14.92 (63.81)	-10.82 (64.76)	24.17 (54.62)	3.82	ns
Cu	55.08^{a} (4.51)	76.25^{b} (1.02)	75.95^{b} (2.25)	29.07	<0.001
Mn	-22.35^{a} (40.61)	30.31^{c} (3.33)	10.33^{b} (5.62)	27.18	<0.001
Mo	1.04^{a} (71.12)	82.01^{b} (0.76)	77.11^{b} (3.56)	56.43	<0.001
Ni	-212.04^{a} (1797.71)	74.64^{b} (1.32)	72.77^{b} (2.24)	32.90	<0.001
Pb	59.08 (68.37)	79.70 (1.71)	70.54 (48.33)	1.44	ns
Zn	54.33^{a} (4.69)	64.62^{b} (1.84)	56.06^{ab} (1.68)	6.41	0.022

Mean ADC for each feed were calculated from three replicates for CON and four replicates for ANA and ALB. Means with the same superscript were not significantly different (Tukey's HSD).

Table 7.8 The effect of the experimental feeds on mean (±SEM, n = 4) crude protein and mineral concentrations (mg kg^{-1}) of carcass samples taken at the end of the growth experiment

Element	Feed			F value	P
	CON	ALB	ANA	(df=2)	
Crude protein	52100 (4400)	52200 (7300)	53100 (3700)	0.04	ns
Ca	16407.66 (1668.39)	15765.74 (2503.62)	15597.46 (1906.96)	0.34	ns
Fe	51.61 (7.61)	48.71 (9.04)	51.92 (12.43)	0.25	ns
K	12162.70 (369.74)	12169.43 (607.77)	12277.02 (407.06)	0.14	ns
Mg	1159.70 (127.25)	1130.45 (102.50)	1148.35 (121.70)	0.12	ns
Na	3209.01 (150.50)	3080.05 (252.13)	3072.91 (225.13)	1.02	ns
P	16583.40 (866.93)	16141.36 (1205.96)	15978.32 (915.87)	0.77	ns
S	18052.67 (2129.01)	18290.18 (1727.41)	18049.22 (1166.27)	0.05	ns

Means with the same superscript were not significantly different (Tukey's HSD).

Table 7.9 The effect of the experimental feeds on mean (±SEM, $n = 4$) trace element concentrations (mg kg^{-1}) of carcass samples taken at the end of the growth experiment

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
B	4.06 (1.16)	4.73 (2.10)	4.24 (1.33)	0.38	ns
Co	0.81[a] (0.56)	1.77[b] (0.25)	0.93[a] (0.29)	14.00	<0.001
Cr	2.01 (2.43)	2.85 (2.79)	1.62 (2.66)	0.46	ns
Cu	2.83 (1.38)	3.06 (1.96)	3.34 (1.23)	0.21	ns
Mn	7.06[a] (1.20)	58.02[b] (19.79)	12.31[a] (1.92)	47.52	<0.001
Mo	0.49 (0.42)	0.66 (0.55)	0.75 (0.37)	0.66	ns
Ni	1.10 (0.77)	0.83 (0.69)	1.17 (0.92)	0.37	ns
Pb	2.46 (1.96)	4.28 (3.57)	1.42 (2.17)	2.37	ns
Se	2.76 (4.58)	5.38 (5.06)	2.60 (3.30)	1.01	ns
Si	18.86 (2.98)	18.02 (0.98)	17.53 (2.49)	0.67	ns
Sr	19.13 (6.32)	17.98 (6.91)	17.83 (7.09)	0.08	ns
V	4.54 (0.18)	4.54 (0.33)	4.60 (0.43)	0.08	ns
Y	25.96 (12.77)	28.28 (11.49)	30.36 (8.50)	0.31	ns
Zn	92.00 (20.48)	100.67 (14.98)	96.76 (29.75)	0.29	ns

Means with the same superscript were not significantly different (Tukey's HSD).

7.3.1.6 Mineral and trace element retention

The CON fed fish had significantly lower retention efficiencies for calcium, iron, sodium, phosphorus, cobalt, copper, yttrium and zinc than the two lupin-based feeds (Table 7.10 and 7.11). Fish fed the ALB feed had significantly lower retention efficiencies for manganese and a greater amount of cadmium than the other two feeds. There was a high degree of variance in the retention of nickel and cadmium in all fish. The apparent retention of the digestible minerals and trace elements showed fewer significant differences in the minerals (Table 7.12) but increases in the significant differences seen in the trace elements (Table 7.13).

7.3.2 Part 2: Maximum voluntary feed intake

The second part of the experiment was conducted between days 75 and 93, and investigated the effect of the experimental feeds on maximum voluntary feed intake and the effect of changing the experimental feed pellet size on feed intake. It allowed comparison of the two methods of measuring feed intake, X-radiography and counting waste feed pellets. The experimental feeds had little effect on maximum voluntary feed intake, as assessed by counting the waste feed pellets. There were no significant differences in maximum voluntary feed intake until day 85, two days after changing to the 3mm feed pellet, with CON having the lowest mean maximum voluntary feed intake and ANA the highest (Figure 7.5). However, pellet size had no significant effect ($P > 0.05$) on maximum voluntary feed intake, as assessed by counting waste feed between days 73-82 and days 83-93.

Table 7.10 The effect of the experimental feeds on mean (±SEM, n = 4) apparent crude protein and mineral retention (%) in whole carcasses of Atlantic salmon

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
Crude protein	21.24 (0.03)	25.32 (0.07)	25.63 (0.03)	0.933	ns
Ca	6.50[a] (0.78)	10.89[ab] (4.04)	11.85[b] (1.86)	4.79	0.038
Fe	1.35[a] (0.25)	3.90[b] (0.82)	3.41[b] (0.47)	23.49	< 0.001
K	12.26 (1.44)	11.99 (1.30)	12.49 (0.26)	0.19	ns
Mg	8.86 (0.73)	9.85 (2.18)	10.01 (0.18)	0.83	ns
Na	5.51[a] (0.31)	8.50[b] (1.40)	8.94[b] (0.53)	17.44	0.001
P	11.00[a] (1.48)	15.99[b] (2.89)	16.70[b] (0.63)	10.74	0.004
S	6.60 (1.91)	9.05 (1.34)	9.08 (0.85)	3.97	ns

Means with the same superscript were not significantly different (Tukey's HSD).

Table 7.11 The effect of the experimental feeds on mean (±SEM, $n = 4$) apparent trace element retention (%) in whole carcasses of Atlantic salmon

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
B	1.98 (1.25)	3.14 (0.51)	1.86 (0.84)	2.46	ns
Cd	19.53^{ab} (20.02)	44.36^{b} (14.52)	-5.39^{a} (24.41)	6.13	0.021
Co	3.17^{a} (6.86)	17.65^{b} (5.77)	17.45^{b} (10.66)	4.25	0.050
Cu	0.96^{a} (0.27)	2.91^{b} (0.45)	2.53^{b} (0.13)	50.29	< 0.001
Mn	1.63^{b} (0.52)	0.86^{a} (0.29)	1.20^{ab} (0.17)	4.26	0.050
Mo	11.39 (12.67)	5.85 (5.10)	6.03 (5.70)	0.53	ns
Ni	52.03 (91.85)	8.05 (11.78)	27.52 (11.79)	0.67	ns
Y	-0.07 (0.25)	0.58 (0.49)	0.58 (0.21)	4.75	0.039
Zn	6.76^{a} (1.73)	18.96^{b} (4.60)	13.44^{b} (2.18)	15.48	0.001

Means with the same superscript were not significantly different (Tukey's HSD).

Table 7.12 The effect of the experimental feeds on mean (±SEM, $n = 4$) apparent retention of the digestible minerals (%) in whole carcasses of Atlantic salmon

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
Ca	12.64 (3.45)	29.19 (5.93)	23.93 (2.98)	1.86	ns
Fe	-25.75 (117.28)	41.72 (15.11)	143.94 (165.82)	3.63	ns
K	24.81 (23.74)	-8.10 (5.15)	-33.33 (41.55)	2.03	ns
Mg	13.92 (1.55)	12.17 (1.40)	12.59 (0.26)	0.63	ns
Na	15.57 (1.27)	13.73 (3.73)	15.58 (1.83)	2.42	ns
P	6.99 (0.54)	9.97 (2.92)	11.24 (2.94)	0.56	ns
S	25.25 (4.94)	26.31 (4.61)	28.55 (3.32)	1.17	ns

Means with the same superscript were not significantly different (Tukey's HSD).

Table 7.13 The effect of the experimental feeds on mean (±SEM, $n = 4$) apparent retention of digestible trace elements (%) in whole carcasses of Atlantic salmon

Element	Feed			F value	*P*
	CON	ALB	ANA	(*df*=2)	
B	3.09 (2.44)	4.78 (0.73)	2.78 (1.36)	1.89	ns
Cd	40.55^{ab} (42.21)	64.25^{b} (18.41)	-7.80^{a} (35.23)	5.16	0.036
Co	6.40 (18.99)	25.81 (15.01)	-12.32 (108.42)	0.31	ns
Cu	1.76^{a} (0.42)	3.82^{b} (0.56)	3.33^{b} (0.05)	23.44	<0.001
Mn	-11.50^{a} (9.16)	2.86^{ab} (0.90)	15.36^{b} (8.68)	12.51	0.003
Mo	114.65^{b} (75.01)	7.05^{a} (6.19)	8.19^{a} (7.68)	8.66	0.010
Ni	-48.78 (82.48)	10.85 (16.19)	37.91 (16.13)	3.46	ns
Zn	12.64^{a} (3.45)	29.19^{b} (5.93)	23.93^{b} (2.98)	12.24	0.004

Means with the same superscript were not significantly different (Tukey's HSD).

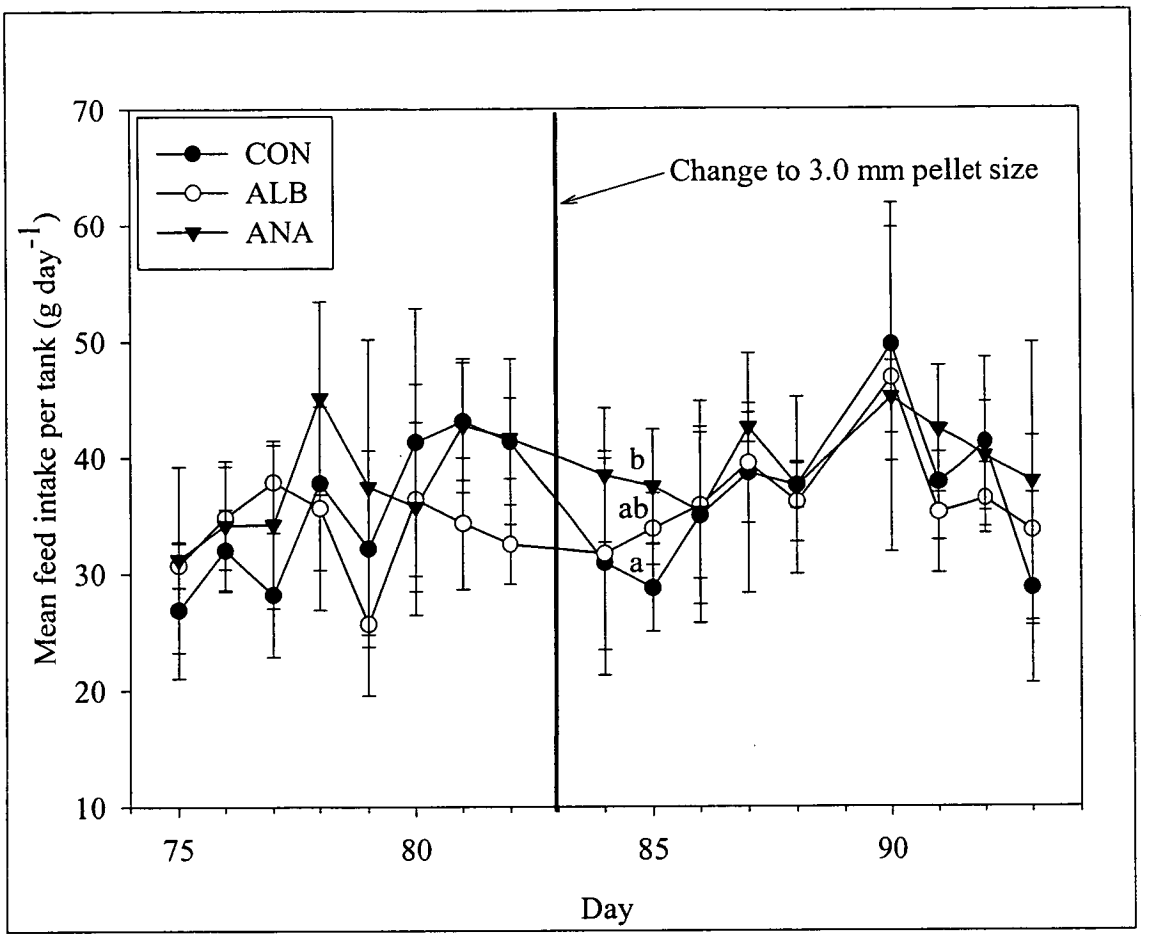

Figure 7.5 The effect of the experimental feeds on mean (±SEM, $n = 4$) feed intake per tank (g day^{-1}), measured by collecting uneaten feed from the outflow, prior to analysis of maximum voluntary feed intake by X-radiography. There was a significant difference in mean feed intake, ($F = 4.850$, $df = 2$, $P = 0.037$).

Maximum voluntary feed intake rose slightly until the end of the experiment when all fish were X-rayed. The only significant difference in feed intake occurred on day 85, two days after changing to the larger feed pellet size.

The X-ray opaque beads were evenly distributed in each of the feeds and there was a strong correlation between the mass of feed and the number of X-ray opaque beads present ($r = 0.892$, $P < 0.001$, $n = 108$). The maximum voluntary feed intake, as measured by X-radiography, differed significantly for each type of feed, but there were no significant differences ($P > 0.05$) in feed intake when measured in terms of mg of maximum voluntary feed intake per g of fish for each tank (Figure 7.6). A comparison of the two methods indicated that maximum voluntary feed intake measured by X-radiography was significantly lower ($F = 8.56$, $P = 0.005$, $n = 36$) than the maximum voluntary feed intake measured by counting waste pellets (Figure 7.7), approximately 25% lower and the difference increased with greater levels of feeding.

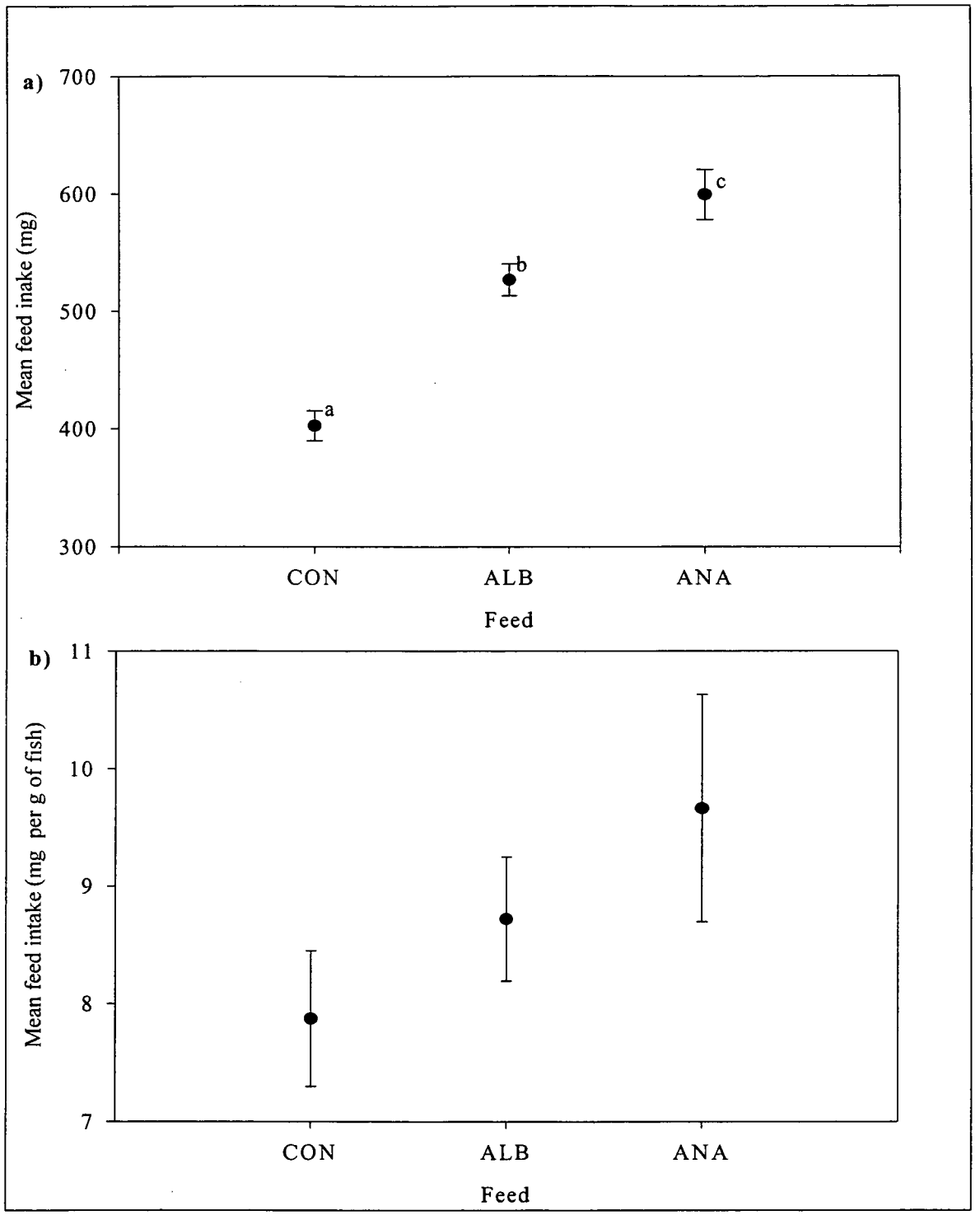

Figure 7.6 The effect of the experimental feeds, as determined by X-radiography, on mean (±SEM, n = 195) a) individual maximum voluntary feed intake and b) maximum voluntary feed intake in terms of mg of feed intake per g of fish. Plots with the same superscript were not significantly different (Tukey's HSD).

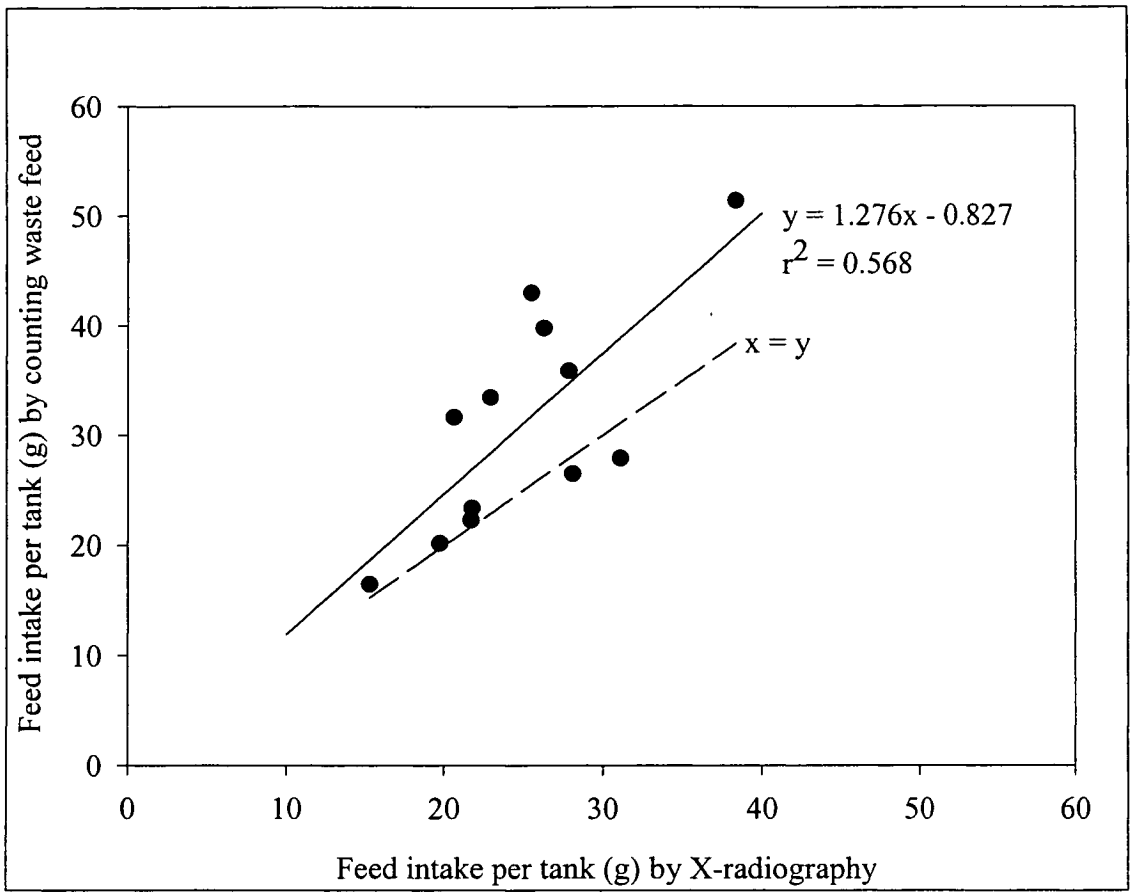

Figure 7.7 Relationship between feed intakes determined by counting waste feed from a tank after feeding and the maximum voluntary feed intake calculated by X-radiography for the same tank one hour after feeding.

7.4 Discussion

7.4.1 Feed parameters

The large differences in manganese concentration between the experimental feeds were a result of differences in manganese content between the two lupin species. These differences in manganese were most likely associated with the growing conditions of the lupins. The mineral content of lupin seeds varies by genotype and environmental conditions (Bhardwaj et al., 1998). Lupins grown in soils with greater concentrations of manganese, or in soils where manganese is more available, due to soil conditions such as pH, can absorb higher concentrations of this element and others (Leeper & Uren, 1993).

7.4.2 Growth performance

There were significant differences in weight gain, specific growth rates, feed consumption and feed efficiency ratios between the control feed and the lupin-based feeds. Fish on all treatments more than doubled their weight over the 64 days of the growth experiment. The feed efficiency ration (FER) of the control feed (0.49) was roughly half of that expected of this reference salmon feed. The FER for the lupin-based feeds (0.59) were less than the efficiencies of 0.78 and 0.97 with 29% and 22% lupin (Carter & Hauler, 2000); 0.91 - 1.00 with up to 50% dehulled lupin (*L. anagustifolius*) in rainbow trout feed (Farhangi & Carter, 2001b), and 1.20 with 20% lupin in an extruded feed (Carter et al., 2002). However, those experiments were conducted under controlled conditions of temperature and photoperiod. The results

from the present experiment confirm that lupin is a viable ingredient for the replacement of protein in fish meal for salmonids, as evidenced by the utilization of macronutrients in trout (de la Higuera et al., 1988; Hughes, 1988, 1991; Morales et al., 1994; Burel et al., 1998; Burel et al., 2000; Farhangi & Carter, 2001a, b; Glencross, 2001; Glencross et al., 2002) and Atlantic salmon (Carter & Hauler, 2000; Carter et al., 2002).

The reduced growth and FERs observed in the fish fed the CON feed may have resulted from a number of factors. It is possible the 208 g kg^{-1} of starch had a significant effect on feed intake, growth parameters, and possibly the digestibility of the fish meal based control feed (Storebakken et al., 1998a). The starch was used to accommodate the inclusion of high levels of the experimental ingredients and balance the carbohydrate content of the lupin ingredients. The CON feed contained crude protein in quantities comparable to the other experimental feeds, and the reference feed used in the preliminary experiment was assessed to have 430.0 ± 0.1 g kg^{-1} DM of digestible crude protein (Chapter 6), comparable to commercial feeds and other experimental controls used in Atlantic salmon research (Carter & Hauler, 2000). This suggests that the quality of fish meal and wheat flour, the major constituents of that reference feed and the CON feed in this experiment, was acceptable. The crude protein of the faecal samples taken from the CON tanks were significantly higher than the other feeds, suggesting that the protein was not digested or there was a significant amount of protein contamination of those faecal samples, possibly from the feed and possible from the sloughing of epithelial cells from the digestive tract. Normally starch levels are kept low in salmon feed due to the limited ability of salmonids to hydrolyse this ingredient (Hemre et al., 1995; Storebakken et al., 2000). Feed

containing greater than 22% starch can limit growth in Atlantic salmon (Hemre et al., 1995), and the control feed contained 20.8% starch. De-hulled *L. albus* and *L. anagustifolius* [Gungurru] are known to contain much less starch, 0.3% and 0.2% respectively, but contain high percentages of non-starch polysaccharides, 32.5% and 35.2% respectively (Allan et al., 2000). Bentonite and α-cellulose can be used as fillers to facilitate formulating experimental feeds that are isonitrogenous and isoenergetic. However, where determining mineral digestibility bentonite is not an appropriate choice, and it would have been impractical to use such a large amount of α-cellulose, as it's relatively low density would have had a major effect on the characteristics of the feed pellets.

7.4.3 ADC

There were a number of differences in mineral and trace element ADC between the present study and ADC from the preliminary assessment of the lupin ingredients (Chapter 6). The negative ADC for nickel and manganese calculated for the CON feed resulted from low concentrations of these elements in that feed, as the faecal output of nickel for all feeds were similar. The ADC of magnesium, manganese, nickel and zinc were greater and the ADC for iron and sodium lower for the lupin ingredients in the current study. There were also differences between the ingredients; *L. anagustifolius* had higher ADC for calcium and phosphorus and *L. albus* and had higher ADC for copper and lower ADC for calcium than the previous experiment. The increases for most of these elements were predictable; the increases in lupin inclusion increased the contribution of more highly digestible amounts of magnesium,

calcium, nickel and zinc to the experimental feeds. However, iron ADC were negative in the present study and lower than predicted, and sulphur ADC significantly higher. It is unlikely that iron contamination of the faecal samples was responsible for these negative values, as the ADC for iron in the CON fed tanks were not affected and were slightly higher than those in the previous experiment (Chapter 6). Non-starch polysaccharides, which negatively affect the digestibility of macronutrients and phosphorus in rainbow trout (Refstie et al., 1999; Glencross et al., 2003), may have antagonistic effects on iron ADC in Atlantic salmon that outweigh the benefits of including greater amounts of the ingredient with increased digestibility. Again the high inclusion of starch in the CON feed may have affected sulphur digestibility. These ADC changes resulting from the effects of antagonistic or anti-nutritional properties identify the problem of basing the inclusion of a novel ingredient on the results obtained from the "standard" 30% replacement assessment of ADC for macronutrients and minerals (Bureau et al., 1999). A factorial design may be more beneficial for determining the effect of ingredients on mineral and trace element nutrition in salmonids (Shearer, 1995).

7.4.4 Retention of minerals and trace elements

Fish fed the ALB and ANA feed retained more minerals and trace elements than those fish fed the CON feed, and there were few differences between the fish fed the lupin based feeds. Fish fed ALB and ANA retained almost twice the amount of calcium, iron and sodium, and 50% more phosphorus than those fed CON. The high proportion of manganese in the ALB feed did not result in increased rates of retention,

verifying the effect of homeostatic regulation of this trace element in salmonids. The high inclusion of starch in the CON feed may have contributed to reduced retention, as it affected the ADC of macronutrients in rainbow trout (Storebakken et al., 1998a). This highlights the need to include commercial feeds as controls within experiments, in addition to isonitrogenous and isoenergetic control feeds. Storebakken et al. (1998b) reported phosphorus retentions of 30% for a fish meal feed which is comparable to those values obtained in this investigation, and suggested that any retention values calculated for waterborne elements, such as magnesium, zinc, and calcium would be biased unless the contribution from water is known. It may not be practical to measure the contribution of water to the retention values by any means other than reporting the total mineral and trace element content available to the fish from the aquatic environment over the period of the experiment. More research is warranted in this particular area of mineral and trace element nutrition, as few studies have explored this topic.

7.4.5 Feed intake

The assessment of the feed intake, as calculated by counting waste feed pellets, during the growth period indicated that the amount of feed actually consumed by the fish may be significantly lower than the estimates used to calculate the FERs, when compared to feed intake as calculated by X-radiography. The fall in feed intake of those fish fed the control feed, may be related to the relatively high inclusion of starch in the feed. Alternatively, the ALB and ANA feeds could have had a positive impact

on feed intake as reported by Carter and Hauler (2000). There seemed to be significantly more faecal output from the lupin feeds, and while feed intake for these feeds may have been greater, this might have been to compensate for greater amounts of indigestible substances in these feeds. In order to compensate for nutritional deficiencies in energy fish may increase feed intake (Carter et al., 2001), and feed intake may also be sensitive to other nutrient. For example, feed intake increases with increases of dietary phosphorus in rainbow trout (Rodehutscord, 1996). The faecal material from the lupin feed was texturally different from the control feed, being composed of relatively large granular components of the lupin grain, and feed particle size is known to affect growth, digestibility (Vielma et al., 1999; Zhu et al., 2001), and gastric emptying (Wankowski & Thorpe, 1979; Sveier et al., 1999) which can occur faster with smaller particles.

When monitoring waste feed to estimate feed intake, Helland et al. (1996) recommend that the recovery rate of each tank be determined and applied to the number of waste pellets recovered. It was not possible to determine the recovery of feed from the experimental tanks in this system. Without fish present in the tanks to increase water movement feed pellets did not exit the tank efficiently, and settled in "dead" zones within each tank, including the space between the outer housing and the inner drain pipe (see Chapter 6, Figure 6.1). It was not possible to apply recovery rates to the measurements of waste feed, and this could account for the difference between the measured feed intake obtained by this method as compared to the X-radiographic technique. Some pellets considered to have been eaten, may have remained in the tank giving a lower measure of the waste feed. This suggests that the method of counting waste pellets overestimated feed intake throughout the growth portion of the

experiment, and the resulting feed efficiency and retention calculations were underestimated. Cold-pelleted feeds disintegrate in a short period of time in water, and although waste pellets were collected and counted immediately after feeding, it is possible that a significant portion of waste feed was not measured. The method of counting waste pellets provided feed intake values 1.27 times higher than those measured by X-radiography (Figure 7.7), suggesting that at least 25% of all waste feed was not accounted for by counting waste feed pellets.

To determine if there were any relationships between feed intake and the ADC of minerals and trace elements correlation coefficients were calculated for each element. There were positive correlations between feed intake and the ADC for boron ($r = 0.686$, $P = 0.020$, $n = 19$), cadmium ($r = 0.749$, $P = 0.008$, $n = 19$), copper ($r = 0.866$, $P = 0.001$, $n = 19$), nickel ($r = 0.669$, $P = 0.024$, $n = 19$) phosphorus ($r = 0.679$, $P = 0.022$, $n = 19$) and sulphur ($r = 0.683$, $P = 0.020$, $n = 19$), and a negative correlation between feed intake and ADC for nickel ($r = -0.707$, $P = 0.15$, $n = 19$). Dietary phosphorus is known to regulate intestinal transport of inorganic phosphate (Avila et al., 2000) and intake in rainbow trout (Rodehutscord, 1996). The significant positive correlations seen in the present study may reflect changes in intake resulting from passive uptake of these elements and effects significant differences in nutrient content on intake.

7.4.6 Maximum voluntary feed intake

There were a number of aspects to the radiographic method that should be noted. Some consideration should be given to the timing of the radiographic procedure. In this experiment two tanks from each feed were measured after the morning or evening feed. This allowed the measurement of feed intake from two meals on the same day, it was not possible to take two x-rays from two meals on one day or to assume glass beads would be present at the end of the day. This was reflected in the difference in the number of many X-ray opaque beads found in the gastrointestinal tract past the stomach in the fish from tanks where the X-rays were conducted in the evening feeding ($F = 423.16$, $df = 1$, $P < 0.001$). There were no differences between the feeds in the number of beads found beyond the stomach in the evening measurements, suggesting similar evacuation rates for all feeds. Considering these facts, a decision was made to use those beads in the stomach for the estimation of feed intake. There was no interaction between time of X-ray (morning or evening) and the type of experimental feed with regard to the maximum voluntary feed intake measurements.

This experiment was not specifically designed to compare ration size and mineral digestibility. Changes can occur in the digestibility of macronutrients with regard to ration size, type of feeding and feed composition in other species of fish (Fernandez et al., 1998). Significant differences in feed intake could have been a factor in differences in mineral and trace element ADC. Future research should consider what effects ration size and the resulting changes in total ingredient input these may have on the apparent digestibility of minerals and trace elements.

7.5 Conclusions

The lupin based feeds provided growth results in Atlantic salmon similar to those obtained in similar research. The *L. anagustifolius* ingredient provided a substantial amount of certain trace elements, such as manganese, and reduced the phosphorus output of the production system, and is a suitable high-protein ingredient for use in salmonid feeds. The lupin feeds significantly increased the retention of iron, sodium, phosphorus, cobalt, and zinc. The need to use relatively large amounts of starch as a filler to produce the control feed may have significantly affected feed intake, growth and mineral and trace element ADC. Future experiments should include a commercial feed as a secondary control to offset the effect of using ingredients such as starch to provide isonitrogenous and isoenergetic feeds for comparison of mineral ADC. The differences in ADC for a number of minerals and trace element confirmed previous assessments of these ingredients at lower inclusion rates, but a number of elements were differed in ADC, possibly due to the antagonistic effects of anti-nutritional compounds such as non-starch polysaccharides.

Maximum voluntary feed intake was not affected by a change in pellet size over time, although there was a significant effect on the day just following the introduction of the new larger pellet size. The assessment of maximum voluntary feed intake by counting waste feed was found to be significantly lower, approximately 25%, than maximum voluntary feed intake as assessed by X-radiography. X-radiographic assessment of feed intake may be a useful method of assessing the recovery of waste feed when tank system designs make such an assessment impractical.

7.6 References

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Chapter 8

General discussion

8.1 Introduction

This study set the groundwork for further research in mineral and trace element nutrition in Atlantic salmon (*Salmo salar*, L.) and other finfish. An effective method of calculating apparent digestibility coefficients (ADC) for minerals and trace elements using common experimental procedures, equipment and fish rearing conditions available to researchers examining Atlantic salmon nutrition was developed (Chapters 2 and 3). Mineral and trace element ADC, the concentrations of these minerals in fish tissues, mineral supplements, dietary supplements (Chapters 4 and 5) and feed ingredients (Chapters 6 and 7) were effectively quantified and used to make inferences about the nutritional effect of aquafeeds and aquafeed ingredients on Atlantic salmon. The outcomes of these experiments identified many factors with significant impacts on determining the mineral status of salmonids, the ADC of minerals and trace elements in aquafeeds as well as areas where further research is warranted.

8.2 Calculating mineral and trace element ADC

Several important factors that affect ADC for minerals and trace elements were identified in this thesis. Marker type, marker concentration, faecal collection, sample processing and analysis presented opportunities for variability and contamination in measuring mineral concentrations used to calculate ADC. There were significant differences in the mineral and trace element ADC determined using yttrium oxide, chromium oxide and acid insoluble ash as digestibility markers. Yttrium oxide at an

inclusion as low as 0.1% had advantages over chromium oxide and AIA for determining mineral and trace element ADC. It was easily decomposed and analysed with ICP-OES and provided ADC with less variance than the other methods (Chapter 2). However, the accurate measurement of "ultra-trace" elements, such as cobalt and molybdenum, may require methods other than the use of digestibility markers and faecal collection. Settlement collection over an 18 h period was the most efficient method of sample collection in fresh water and produced the greatest quantity of faecal material, without disturbing feeding habits. However, settlement of faeces allows leaching or contamination of faecal samples with minerals, and it is not practical to use this method in sea cages. Therefore, stripping is the preferred method of collecting faeces in a commercial production setting. Stripping provided ADC for minerals significantly lower than those obtained with settlement collection, limited leaching and contamination, and required handling of fish that may affect ADC if repeated sampling is required (Hajen et al., 1993a; Vandenberg & de la Noüe, 2001).

The ADC values from the reference feed used in this thesis characterised mineral and trace element digestibility of minerals in a fish meal based feed (Table 8.1). Potassium had the smallest standard deviation of all the elements measured, and routinely provided ADC of 99.6%. These high ADC are likely a result of the solubility of potassium and may not reflect true digestibility. The lack of variance in ADC and the fact that potassium is found in most feed and faecal samples in relatively large concentrations, as compared to other minerals, made this element a useful check of analytical procedure and sample processing. The use of potassium as an internal digestibility marker warrants further research. Sodium and sulphur also

Table 8.1 Means and the standard error of the means (n = 96) of ADC (%) for minerals and trace elements calculated from several experiments using the fish meal based reference feed.

Element	Mean	SEM
K	99.62	0.46
Na	89.29	4.53
S	82.20	6.21
Ni	76.46	110.03
P	54.02	7.75
Cu	48.33	12.80
Mo	41.22	164.59
Zn	40.88	12.38
Co	38.31	21.38
Mg	36.78	15.07
Mn	5.42	14.67
Ca	0.72	23.82
Fe	-2.19	17.27
Si	-24.98	36.66
Cd	-29.12	83.56
B	-73.40	131.03
Al	-364.40	372.80

These ADC were calculated from faecal samples collected via settlement (18 h) from juvenile Atlantic salmon of less than 200 g, maintained in recirculating fresh water systems, fed the reference salmon feed, composed primarily of fish meal (70%) and wheat flour (15%). The external digestibility marker was yttrium oxide included in the feed at 0.1%. Only those elements which were consistently measurable over all experiments were reported.

displayed relatively stable, high ADC. The ADC for phosphorus was equivalent to, or slightly higher than, values reported for fish meal based feeds in other research (Riche & Brown, 1996; Sugiura et al., 2000b; Thodesen et al., 2001), and the higher values may have resulted from differences in faecal collection methods. The trace elements zinc, copper and manganese produced reliable ADC, but nickel, molybdenum, boron and aluminium were highly variable.

The methods of decomposition and analysis of feed and faecal samples for minerals and trace elements vary greatly and can impact ADC calculations. Wet decomposition with concentrated nitric acid, in open containers, at low temperatures (100 °C), effectively decomposed aquafeeds and faecal samples. Combustion, including dry ashing, of samples at high temperatures, in conventional or microwave ovens, can lead to losses of zinc, calcium, iron, chromium and copper, due to volatilization (Vandecasteele & Block, 1993). The addition of hydrogen peroxide or the use of perchloric acid proved to be of limited benefit, particularly considering the risks of explosions involved when using perchloric acid. Inductively coupled plasma optical emission spectrophotometry analysis provided a full range of mineral and trace elements quickly from relatively small samples with the sample processing described above. However, it was not always possible to detect trace elements such as arsenic, lead, cadmium, molybdenum, nickel, selenium and tin in faecal samples, but the method used could be altered to look specifically at these elements if required, by decreasing dilution factors, and alternative methods of analysis for these elements exists (Underwood, 1971).

There is a need for standardisation of sample collection, processing, sample analysis and calculations of ADC in aquaculture experiments attempting to identify the digestibility, retention and deficiency effects of minerals and trace elements. Sampling techniques, processing, and analytical methods vary considerably, making it difficult to compare ADC results obtained under differing conditions. Settlement collection has gained in popularity when working with smaller, quickly growing fish, housed in tanks, and stripping is the desired method of faecal collection with large fish held in sea cages (see Chapter 1, Table 1.3). The application of standard methods for calculating ADC would be of great benefit to the research community and aquafeed producers. Standardised methods and the use of the same reference ingredients or diets between locations, would limit any effects resulting from differences in experimental methods.

ADC are only apparent digestibility coefficients of minerals and trace elements and do not reflect the true digestibility of an element from a feed or ingredient. Waterborne elements, such as zinc (Lovegrove & Eddy, 1982) copper (Waiwood & Beamish, 1978), can be taken up by fish and excreted in the faeces, lowering ADC. The ADC calculated for aluminium, boron, silicon, cadmium, iron and calcium were often negative indicating that these elements were either consumed by drinking or taken up through the gills. Contamination is also a concern when collecting samples for trace element analysis. The nickel digestibility of the soybean product analysed in Chapter 6 may indicate an effect of ingredient processing, however, no information is available regarding the level of contamination that occurs from these routes. Two main sources of variability in ADC arising from fish are genetic differences and total feed intake. Thodesen et al. (2001) identified a significant differences in the

absorption of potassium, calcium, magnesium, zinc, sodium and iron attributed to genotypic differences in Atlantic salmon, possibly connected to drinking rate, active absorption of nutrients, and excretion mechanisms. The digestibility of energy and protein differs between species of salmon (NRC, 1993), as does phosphorus (Lall, 1991), but it remains to be determined if differences exist for other elements. Sugiura et al. (1998a) found positive correlations between the total intake of an element and the apparent availability of potassium, sodium and zinc in salmonids. It may never be possible to accurately measure the true digestibility of minerals in finfish considering the difficulties in obtaining and measuring all routes of mineral excretion and intake without altering the metabolic activity of the fish in the process. It may be more advantageous to look at the bioavailability of minerals in an ingredient using other means, such as protein products that require minerals as cofactors in enzymatic processes and in non-enzymatic structural units (Watanabe et al., 1997). Therefore, mineral retention was an important indicator of bioavailability used to assess digestibility.

8.3 Effects of supplementation

Mineral supplementation affected mineral and trace element ADC, and the concentration of minerals in tissues over time. Significant changes were also observed in mineral and trace element ADC over several weeks. This reinforces concerns regarding when faecal collection should be conducted after starting a new feed. Sugiura et al. (2000a) suggest collections should occur quickly after starting a new feed, to prevent false ADC calculations resulting from acclimation to dietary

minerals through homeostatic mechanisms. The relationship between mineral and trace element ADC taken in short term experiments and the effects of using that feed over longer periods need to be investigated to identify other factors involved in the relationship to prevent nutritional deficiencies and improve the effectiveness of ADC assessment. Tissue samples provided greater accuracy for identifying differences in the mineral nutrition status of salmon for selenium, zinc and copper, although there were few correlations between ADC and the mineral content of these tissue samples (Chapter 4). Mineral and trace element concentrations in muscle, blood, and liver-kidney samples did not always correlate to the significantly different ADC observed over the same period for those elements. The use of common feed ingredients rather than purified or semi-purified ingredients revealed the effects of feed consistency on the variability of mineral concentration within feed samples.

Citric acid supplementation, when combined with mineral supplements, improved the ADC of calcium, magnesium, potassium, phosphorus and sulphur in Atlantic salmon. No apparent effect of citric acid supplementation on the mineral or trace element concentrations was seen in whole blood 14 days after the start of supplementation, and did not provide a useful measure of mineral bioavailability in the short term. ADC effectively measured the effect of citric acid supplementation after just 14 days, but longer term investigations using this dietary supplement may provide further information of its effect on mineral stores in the body.

8.4 Effects of ingredients

Initial assessment of high-protein, fish meal replacement ingredients, including two lupins and a soybean protein concentrate, indicated that the ingredients were equivalent in the provision of digestible energy and crude protein, but provide significantly different concentrations of all minerals and most trace elements between species and cultivars. These high-protein ingredients that could replace a proportion of fish meal and increase the sustainability of aquaculture feeds and reduce the environmental impacts of feeds. The mineral and trace element variability within the major ingredients of aquafeeds, such as fish meal and wheat flour, limited the accuracy of the ADC values calculated using reference feeds composed of these common ingredients. Alternative methods of determining the ADC of minerals and trace element in ingredients that are so dissimilar in nutrient concentration from the reference feed may be required. Mineral retention and other measures of bioavailability may be employed to determine the mineral provision of ingredients in aquafeeds. Replacing 44% of the protein content in a salmonid feed with lupins produced growth results in Atlantic salmon similar to those obtained in other research, provided a substantial amount of certain trace elements, such as manganese, and reduced the phosphorus output of the production system (Chapter 7).

ADC for single ingredients should only be calculated with a formula that accounts for the nutrient provision of that ingredient to the experimental feed. An evaluation of the two different formulas widely used to calculate ADC in single ingredients by Forster (1999) indicated that unless the nutrient contribution of an ingredient is

accounted for in the formula, errors in ADC calculations will occur. This consideration is vital for trace elements such as manganese, which had concentration was up to 50 times greater concentrations than in fish meal (Chapter 7). The lupin-based feeds increased the retention of iron, sodium, phosphorus, cobalt, and zinc.

Antagonistic and synergistic effects from substances in the ingredients produced differences in the mineral and trace element ADC, including a fish meal based control feed which contained relatively large amounts of starch as filler. The starch and the presence of non-starch polysaccharides in the lupin feeds may have significantly affected feed intake, growth and mineral and trace element ADC. Commercial feeds should be included in experiments to provide an alternative control, when such high levels of starch need to be employed to provide isonitrogenous and isoenergetic feeds for comparison of mineral ADC.

Feed intake in finfish is an area that has been explored in some depth, particularly with regard to macronutrients (McCarthy et al., 1993; Helland et al., 1996; Carter et al., 2001; de la Higuera, 2001; Jobling et al., 2001), however, little is known of the effect of intake on mineral and trace element nutrition (Medale et al., 1998). The subject was explored briefly in this thesis, but further work is required to assess the impact of intake on mineral digestibility and nutrition in salmonids. The method of feed intake assessment needs to be considered carefully with regard to the type of system used, and the impact of measurement on the fish. Assessment of the effect of intake on phosphorus digestibility and retention in salmonids is ongoing at this facility and elsewhere (Apines et al., 2003).

8.5 Current and future research

There are many additional factors that will need to be explored in relation to mineral and trace element digestibility. Ration size (Fernandez et al., 1998), temperature (Bendiksen et al., 2002), differences between triploids and diploids and feed processing (Pongmaneerat & Watanabe, 1993) affect growth and the digestibility of macronutrients, and there may be mineral components of these effects. Current research is exploring the effects that novel feed ingredients (Cheng & Hardy, 2002) and vitamins (Vielma et al., 1999; Graff et al., 2002a; 2002b) have on mineral and trace element digestibility. The ratio of digestible protein to digestible energy in a feed and how this ratio should be changed over a production cycle and with environmental conditions, are sighted as crucial for meeting the requirements of essential nutrients under a given production system (Kaushik & Medale, 1994). This concept should be applied to mineral and trace element supplementation, and varied over the production cycle. Finally, the digestibility of minerals and trace from major ingredients should be part of the nutritional considerations in formulating feed at least cost and maximum efficiency, reducing the cost associated with mineral pre-mixes and over-supplementation.

Continued research is required to derive ADC for different production systems, rearing environments and fish species. Most of the information available on dietary nutrient requirement has been determined on laboratory-based experiments, conducted in artificial tanks, using species cultivated by industrialised countries, in temperate climates, such as salmonids (Tacon, 1988). However, much of the aquaculture growth that is occurring in developing countries is tropical and

subtropical semi-intensive and extensive pond productions systems (Naylor et al., 2000). While digestibility information derived for the formulation of complete pelleted rations for use in developed countries is economically and environmentally beneficial, this information may not be applied to "less developed" production systems (Tisdell, 1999). However, the same potential for economic and environmental benefits exist (New, 1996; McIntosh, 2002) and should be achieved improving the sustainability of emerging aquaculture ventures.

8.6 Overall Summary

The most important findings form this study on mineral and trace element nutrition in salmonids were:

- The settlement method of faecal collection provided the required amount of sample for mineral and trace element analyses.
- The stripping and dissection methods of faecal collection limited the leaching of and the contamination of faecal samples with minerals and trace elements.
- Yttrium oxide, included at a concentration of 100 mg kg^{-1} or 0.1% of the total feed was the most effective digestibility marker for assessing mineral and trace element ADC.
- Chromium oxide was not effectively decomposed by concentrated nitric acid, and was therefore, not useful as an external marker for assessing the digestibility of minerals and trace elements.

- Acid insoluble ash was effectively used to assess the ADC of minerals and trace element, although the amounts of faecal material required were at least 4 – 5 times that required using yttrium oxide.
- ADC for some elements change over a 24 hour period, and collecting faeces via settlement for a period of 18 hours after feeding provided accurate, representative ADC for all elements assessed.
- Potassium in fish meal based feeds had the most consistent and highest ADC of any element analysed (99.62 ± 0.46%).
- Significant differences in mineral and trace element concentrations found in the various tissues sampled did not always correlate to the significantly different ADC observed over the same period for those elements.
- Tissue samples provided stronger statistical power for identifying small differences between elements such as: copper, iron, selenium, and zinc.
- Citric acid supplementation improved the ADC of potassium and sulphur, and when combined with mineral supplements improved the ADC of aluminium, chromium, molybdenum, nickel, calcium, magnesium and phosphorus.
- Whole blood samples provided a useful measure of the effect of citric acid supplementation on mineral bioavailability in the short term.
- There was no apparent effect of citric acid supplementation on the mineral or trace element concentrations of whole blood on samples taken 14 days after the start of supplementation.
- White lupin (*Lupinus albus*), Australian sweet lupin (*Lupinus anagustifolius* [Gungurru cultivar]) and soybean (*Glycine max*) provided acceptable amounts of digestible protein and energy, were effective replacements for fish meal and did not limit the digestibility of minerals or trace elements.

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Appendices

Appendix A: Definitions of standard terms

Acid insoluble ash (AIA):

The amount of inorganic material remaining in a sample after ashing at high temperature (550 °C) and decomposition with concentrated acid. The method described by Atkinson et al. (1984) was used to determine the AIA content of faecal and feed samples in this thesis.

Apparent digestibility coefficient (ADC):

A coefficient (%) describing the amount of nutrient absorbed by a fish from a feed, calculated by comparing the concentration of an indigestible marker present in the feed to the concentration present in the faeces. ADC were calculated, for each mineral and trace element that was present in reportable concentrations, according to the following equation from Maynard and Loosli (1969):

$$ADC(\%) = 100 - \left[\frac{[M]feed \times [N]faeces}{[M]faeces \times [N]feed}\right] \times 100$$

where $[M]$ is the concentration (mg kg^{-1}), of the digestibility marker, and $[N]$ the concentration of the nutrient (mg kg^{-1}).

Apparent mineral retention:

The change in the amount of mineral content of a fish resulting from the amount of total mineral provided to the fish in feed during that time. The apparent retention (%) was determined according to Storebakken et al. (1998):

$$Apparent\ retention = 100 \times \left[\frac{\left(BW_f \times [N]_f\right) - \left(BW_i \times [N]_i\right)}{\left(Feed \times [N]_{Feed}\right)} \right]$$

where BW_f is the final body weight of the fish, $[N]_f$ is the final concentration of nutrient (mg $fish^{-1}$) in the carcass; BW_i is the initial body weight of the fish; $[N]_i$ is the initial concentration of nutrient in the carcass (mg $fish^{-1}$); Feed is the total amount of feed consumed (mg) by the fish over that period; and $[N]_{Feed}$ is the concentration (mg) of the nutrient provided to the fish over that time.

Blank subtraction:

An analytical and mathematical method whereby the mean elemental concentration measured in a decomposed sample consisting only of the reagents used in its preparation (acids, water etc.) is subtracted from a sample prepared in the same fashion.

Decomposition:

Separation of a sample into its constituents by chemical reactions with concentrated acid. As this process is often referred to as digestion, to limit confusion the term decomposition was used when discussing this procedure.

Decomposition matrix effect:

The effect of substances in a sample resulting from the decomposition process that may alter the spectral emissions during sample volatilization.

Inductively coupled plasma optical emission spectrometry (ICP-OES):

A plasma (a hot gaseous cloud of Argon, T>7500K) based technique, where liquid (decomposed) samples are nebulized into the plasma, the samples are decomposed to atoms and ions, and the light emitted measured and compared to independently prepared standard solutions. Detection of a number of elements is possible simultaneously, with detection limits of the order of 0.05 ppm.

Interference (spectrographic):

A spectral overlap from another element or matrix constituent, resulting in an incorrect concentration (of the element of interest) being measured. The matrix constituents that could cause interference in aquatic samples, include, organic matter, and borosilicates from the decomposition glassware. Carbon, phosphorus and calcium were the elements considered to contribute most to interference, and samples were diluted in an attempt to limit their effects.

Marker recovery:

The quantity (%) of indigestible marker measured by analytical means in a feed compared to the amount included.

Minerals:

Elements found in concentrations greater than 100 mg kg^{-1}, including;

calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), and sulphur (S).

Quality control sample (Dogfish muscle):

A sample of known elemental composition (both type and concentration) analysed to help ascertain the accuracy (or otherwise) of the analytical process. Dogfish muscle (DORM-2, National Research Council, Canada) were included as quality control samples in decomposition procedures.

Trace element:

Elements found in concentrations of less than 100 mg kg^{-1} in a biological sample, including: aluminium (Al), arsenic (As), boron (B), barium (Ba), cadmium (Cd), cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), selenium (Se), silicon (Si), tin (Sn), vanadium (V), and zinc (Zn).

Appendix A references:

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Appendix B: Chapter 2

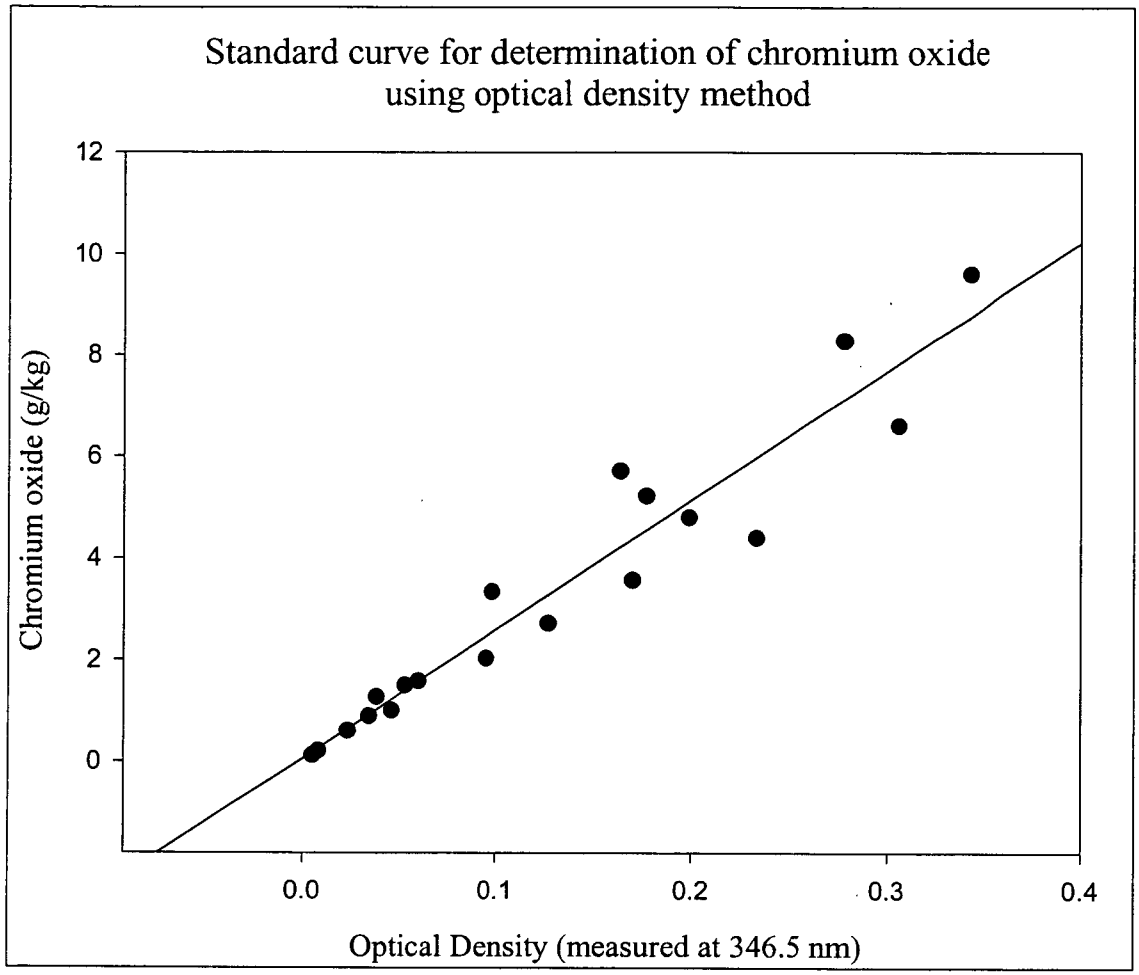

Figure B.1 The standard curve used to calculate the concentration of chromium in faecal and feed samples from absorption measurements in samples decomposed in perchloric acid. Each point represents the optical density calculated for a known quantity for chromium oxide, and produced the following equation used to calculate chromium in samples: chromic oxide ($g\ kg^{-1}$) = 21.486x, (r^2 = 0.9829) where x = optical density of a sample measured at 346.5 nm.

Table B.1 Mineral ADC (%) from Atlantic salmon by sampling period (6-h, 12-h and 24-h).

Element	Sampling period (h)							F value	*P*
	0-6	6-12	12-18	18-24	0-12	12-24	0-24	(*df*=6)	
Ca	24.15^{ab} (2.90)	28.71^{b} (1.99)	23.37^{ab} (1.50)	13.72^{a} (3.41)	26.88^{b} (2.10)	21.60^{ab} (4.73)	24.56^{ab} (5.36)	3.29	0.011
Fe	-37.46^{a} (2.80)	-6.45^{bc} (7.95)	4.54^{c} (10.65)	2.59^{c} (3.40)	-16.05^{b} (4.72)	-2.76^{bc} (14.35)	-9.77^{bc} (8.56)	12.32	<0.001
K	99.48 (0.00)	99.57 (0.00)	99.70 (0.00)	99.71 (0.00)	99.46 (0.00)	99.51 (0.00)	98.97 (0.17)	1.02	ns
Mg	60.88^{d} (1.60)	49.47^{c} (1.04)	36.34^{b} (1.83)	23.29^{a} (1.52)	53.55^{cd} (0.29)	24.79^{a} (3.81)	39.37^{b} (0.94)	60.30	<0.001
Na	85.96 (0.22)	86.58 (0.13)	87.88 (0.25)	88.20 (0.25)	83.13 (1.65)	84.93 (1.64)	82.82 (1.02)	2.87	ns
P	63.43^{c} (1.44)	65.14^{c} (0.46)	62.98^{bc} (0.97)	54.09^{a} (0.88)	62.14^{bc} (0.99)	56.40^{ab} (1.54)	59.78^{abc} (1.34)	7.08	<0.001
S	79.76^{ab} (0.19)	83.36^{b} (0.11)	83.01^{ab} (0.28)	81.14^{ab} (0.35)	79.33^{ab} (1.16)	79.09^{ab} (1.29)	77.67^{a} (1.25)	3.07	0.015

Means (± SEM, $n = 6$) with the same superscript were not significantly different (Tukey's HSD).

Table B.2 Trace element ADC (%) from Atlantic salmon by sampling period (6-h, 12-h and 24-h).

Element	Sampling period (h)							F value	*P*
	0-6	6-12	12-18	18-24	0-12	12-24	0-24	(*df*=6)	
Al	-255.57^{b} (946.72)	-445.65^{ab} (4205.55)	-654.09^{a} (7881.16)	-332.98^{ab} (2443.32)	-215.17^{b} (3142.08)	-311.61^{ab} (853.45)	-177.44^{b} (2902.38)	4.24	0.002
B	-73.70 (353.97)	-131.83 (295.14)	-233.82 (3501.59)	-44.55 (261.52)	-132.32 (3116.02)	-134.61 (307.93)	-87.01 (909.21)	1.51	ns
Ba	2.29^{ab} (4.03)	2.35^{ab} (21.43)	1.60^{ab} (4.42)	-10.51^{a} (7.23)	7.00^{b} (0.82)	-6.22^{ab} (4.56)	3.34^{ab} (3.68)	2.87	0.021
Cd	-5.02^{b} (3.99)	-5.38^{b} (7.09)	-20.79^{ab} (7.24)	-32.71^{a} (8.71)	-9.23^{b} (8.03)	-28.93^{a} (6.51)	-20.70^{ab} (8.42)	8.55	<0.001
Co	22.74^{a} (13.77)	46.65^{b} (11.67)	35.48^{ab} (6.57)	28.65^{ab} (22.52)	25.77^{ab} (11.04)	25.38^{ab} (8.77)	25.14^{ab} (22.58)	2.42	0.044
Cu	45.78 (1.85)	53.04 (1.51)	48.89 (0.85)	48.46 (1.18)	50.84 (10.91)	42.00 (6.33)	43.38 (2.86)	2.16	ns
Mn	20.72^{e} (3.45)	14.57^{cde} (3.62)	4.06^{abc} (6.24)	-5.31^{a} (2.69)	19.02^{de} (0.59)	-3.03^{ab} (3.45)	8.39^{bcd} (3.96)	15.12	<0.001
Mo	12.13 (116.22)	-48.30 (1592.79)	5.07 (877.21)	-2.48 (403.08)	-30.06 (408.40)	-38.95 (584.66)	-22.36 (247.58)	0.44	ns
Si	-53.23 (90.10)	-48.66 (109.75)	-54.43 (124.02)	-45.92 (126.21)	-30.17 (138.92)	-33.73 (108.91)	-22.10 (205.21)	0.60	ns
V	-51.30 (5060)	-42.99 (2557)	-100.95 (3642)	-11.51 (2428)	-68.37 (1874)	-150.08 (2317)	-8.20 (3264)	0.44	ns
Zn	49.15 (6.11)	48.64 (3.02)	43.66 (6.26)	32:09 (17.00)	47.09 (2.91)	48.57 (38.76)	43.57 (2.15)	1.46	ns

Means (± SEM, n = 6) with the same superscript were not significantly different (Tukey's HSD).

Table B.3 Mineral ADC (%) by marker concentration as determined by chromium oxide (ICP-OES).

Element	Chromium concentration				F value	*P*
	1.0%	0.1%	0.01%	0.00 1%	(*df*=3)	
Ca	-24.36[a] (35.07)	63.88[c] (9.65)	50.78[bc] (16.69)	28.74[b] (43.61)	25.32	<0.001
Fe	-4.75[a] (7.15)	73.04[b] (5.55)	53.74[b] (21.20)	52.67[b] (16.98)	40.64	<0.001
K	99.76 (0.00)	99.91 (0.00)	99.89 (0.00)	99.84 (0.00)	3.55	ns
Mg	5.57[a] (49.34)	72.30[b] (10.12)	60.91[b] (7.44)	46.92[b] (24.90)	17.32	<0.001
Na	92.22[a] (1.36)	97.62[b] (0.09)	96.08[ab] (0.16)	94.85[ab] (0.33)	5.22	0.010
P	42.42[a] (8.43)	83.30[b] (2.75)	76.10[b] (3.04)	69.58[b] (7.79)	26.20	<0.001
S	73.01[a] (2.03)	92.38[c] (0.62)	88.66[bc] (0.91)	85.66[b] (1.73)	24.50	<0.001

Means (± SEM, $n = 6$) with the same superscript were not significantly different (Tukey's HSD).

Table B.4 Trace element ADC (%) by marker concentration as determined by chromium oxide (ICP-OES).

Element	Chromium concentration				F value	*P*
	1.0%	0.1%	0.01%	0.001%	(*df*=3)	
Ba	-34.36[a] (44.81)	67.25[b] (4.39)	47.73[b] (36.68)	40.06[b] (30.82)	32.00	<0.001
Cd	-49.25[a] (84.30)	73.13[c] (6.56)	27.06[bc] (65.22)	61.11[b] (19.89)	35.45	<0.001
Co	62.21[a] (8.60)	95.90[c] (0.08)	82.82[bc] (8.67)	68.39[ab] (13.70)	13.04	<0.001
Cu	33.34[a] (23.81)	77.82[b] (7.81)	68.32[b] (15.78)	62.40[b] (27.62)	7.00	0.003
Mn	-17.02[a] (23.11)	68.57[b] (7.81)	52.71[b] (15.78)	44.60[b] (27.62)	33.98	<0.001
Se	-105.27 (1197.56)	-45.07 (325.01)	-35.63 (419.27)	-421.38 (19934.83)	2.52	ns
Si	-1.72[a] (217.52)	75.08[b] (3.88)	51.51[b] (72.93)	42.46[ab] (34.20)	6.34	0.004
Y	-239.03[a] (3417.39)	40.21[b] (16.92)	18.92[b] (956.03)	50.56[b] (46.55)	9.33	0.001
Zn	-239.03[a] (8.49	40.21[b] (3.42)	18.92[b] (32.14)	50.56[b] (11.37)	17.53	<0.001

Means (± SEM, $n = 6$) with the same superscript were not significantly different (Tukey's HSD).

Appendix C: Chapter 3

Table C.1 The acid insoluble ash (AIA) content of feed and faecal samples obtained via settlement collection

Sample	Sample mass (g)	AIA (mg kg^{-1})
Feed	9.02	2058.43
Feed	11.94	1790.97
Feed	11.04	2261.85
Feed	12.93	2222.91
Faeces	2.71	4941.34
Faeces	4.51	4917.71
Faeces	3.18	4583.39
Faeces	3.38	5575.77
Faeces	1.89	4646.70
Faeces	3.27	5633.03
Faeces	2.60	5533.50

Appendix D: Chapter 6

Table D.1 The ingredient data calculated in this experiment

Ingredient	**Lupin**	**Lupin**	**Soybean**
Variety	Dehulled lupin meal (*Lupinus anaustifolius* [Gungurru cultivar])	Dehulled lupin meal (*Lupinus albus*)	Soybean concentrate (*Glycine max*) Soycomil® P
Source	M.C. Croker, NSW	M.C. Croker, NSW	Gibson's (ADM), NSW
Fish species used	Atlantic salmon	Atlantic salmon	Atlantic salmon
Proximate (as is) (g/kg)			
DM	950	950	960
CP (N * 6.25)	355	360	600
Digestible CP	328	364	385
Crude lipid	66	60	58
Energy (MJ kg^{-1})	19.33	18.89	17.82
Digestible energy	14.61	15.97	15.11
Mineral content (mg kg^{-1})			
Ca	1569	1080	5518
Fe	42	38	122
K	10297	11214	23320
Mg	1946	1190	3941
Na	61	122	21
P	3882	4096	9537
S	3025	2839	5573
Trace element content (mg kg^{-1})			
Al	3.31	8.36	4.85
As	0.89	1.36	1.50
B	19.46	19.44	28.92
Co	0.09	0.72	0.03
Cu	5.38	8.82	15.10
Mn	235.44	3644.26	52.61
Mo	4.42	29.87	1.71
Ni	2.15	3.82	0.33
Si	3.61	4.39	3.48
Se	0.80	1.24	3.41
Sr	8.37	4.29	6.18
V	11.16	9.07	21.81
Zn	35.64	42.05	37.79